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Method and Test Kit for Determining the Amounts of Individual Polynucleotides in a Mixture

The Technical Field of the Invention

The present invention is related to a method and a test kit for determining the amounts or relative proportions of more than one individual polynucleotide sequence present in a mixture of polynucleotide sequences. The method and the test kit enable the determination of dynamic variations of individual organisms or subpopulations present in a sample containing a mixture of organisms, i.e. a target population. The invention is based on a quantitative affinity aided solution hybridization determination of polynucleotide sequences and is closely related to the International patent application PCT/FI02/00023 in which the probes have distinct and distinguishable size. Unlike this the probes of the present invention are of approximately the same sizes. The method and the test kit are useful in health care, environmental research, pharmaceutical industry and food industry and are applicable for many other diagnostic, biotechnical and scientific purposes.

The Background of the Invention

The rapidly accumulating genetic information combined with combinatorial chemistry and bioinformatics allowing the handling of enormous amounts of information has created a demand for new more accurate methods, which allow the simultaneous and/or sequential studies of dynamic situations and variations in natural environments. Accordingly, totally new approaches for carrying out research in molecular biology, health care, epidemiological studies, pharmaceutical and food industry are required.

In health care as well as in pharmaceutical and food industry, especially, practicing physicians, environmental consults, industrial hygienists, safety officers, health inspectors, environmental consults, veterinarians and/or other persons working with or being responsible for the evaluations of possible health or epidemiologic risks have a need for new effective tools for assessing the effects of remedial, sanitary or other measures on whole populations of organisms. There is for example an increasing demand for methods and tools for assessing the effects of new and conventional treatment modalities, including sanitary and remedial measures.

Based on the accumulating information including availability of genetical key elements and the knowledge of their biological role and functions, new methods are continuously developed. A powerful new tool is the oligomer-chip technology. The common characteristic of the microarray techniques and the feature distinguishing it from the present invention is that the probes, i.e. the polynucleotide sequences used as reagents or probes are immobilized or coupled to a solid carrier. The immobilization of the probes acts as a steric hindrance and prevents the hybridization to take place in a stochiometric fashion resulting in low yield. Even if the oligomer-chip technology allows simultaneous handling of an enormous amount of samples, the results does not allow quantitative comparison in a wide dynamic range.

In the Finnish patent application 20010041 which corresponds to the International Patent Application PCT/FI02/00023 a method and test kit for overcoming the problem of obtaining quantitative results is described. Said patent applications disclose a method and a test kit, including the reagents for quantitative determination of polynucleotides or variations in their amounts in a cell or tissue sample using organized pools of soluble polynucleotide probes with distinct sizes varying from 16 to several thousands of base pairs. The quantitative method allows comparative assessment of variations, e.g. in transcription profiles or expression patterns. Said method is based on the varying and distinct sizes of soluble polynucleotide probes. It is the difference in size of the probes that enables the assessment of the individual nucleic acid sequences. Probes from more or less conserved or hypervariable regions are known to enable classification and organization of different organisms in phylogenetic levels including groups, genus, species or subspecies. A quantitative evaluation of the amounts of individual organisms, their subpopulations in a mixture using said probes would enable studies of dynamic variation in target populations. Such evaluations would have several useful applications. Unfortunately, the method disclosed in WO/FI02/00023 is not applicable to said probes because they are oligonucleotide sequences having approximately the same size and sufficient resolution for reading the results cannot be achieved.

Consequently, the objective of the present invention is to provide a new and effective tool for the specialists working with or being responsible for investigations and evaluations of possible health risks and the need of repair or other remedial measures.

The objective of the present invention is to provide a method and test kits not only for determining the relative amounts of individual organisms, or certain subgroups in a population, it also allows comparative assessments of sequential time variations in the

population due to internal or selected applied measures or interventions or comparative assessment of population in samples obtained from different sites. Simultaneously, the objective is to provide a very sensitive test, which allows the quantitative determination of very small amounts of analyte polynucleotides, which otherwise would be under the detection limit.

The advantages related to the present invention as well as to the method and test kits described in PCT/FI02/00023, include the fact that the quality of the polynucleotide preparation, especially RNA to be analyzed, is not critical. For example, RNA known to require special treatment due to its instability, can be used directly for the quantitative assessment. The manufacturing of test kits, which need not include immobilization steps and certain commercially available reagents allows preparation of easily adaptable tailor-made tests, directing the attention to certain subsets of genes in a given organism.

The method can be used as fully automatic or semiautomatic assemblies. The procedure can be interrupted at several stages. The samples and reaction products can be preserved until sufficient data has been collected or it is more convenient to continue the process, e.g. recording the results.

A Summary of the Invention

As a summary, the present invention allows a simultaneous, quantitative recording of changes and variations of the amounts and/or relative proportions of more than one individual polynucleotide in a mixture. The method and the test kit enable the determination of amounts and/or relative proportions of individual organisms or subgroups thereof in a mixed population pool, which have been taken e.g. at different points of time or from different sites. This is useful especially when studying the effects and the impact of various physical and chemical stimuli applied on the population, including antibiotic treatment, hygienic measure and other interventions and also allows the evaluation of inherent changes in population. The invention allows simultaneous comparative assessment of several biological phenomenons.

The method and test kit of the present invention are not only quantitative, they can also be made very sensitive and allow quantitative detection polynucleotide sequences present in diminutive amounts. The characteristic features of the method and test kit of the present invention as well as their applications are as defined in the claims.

A Short Description of the Drawings

Fig. 1 shows the separation of single stranded DNA fragments and oligonucleotides with different fluorophores by capillary electrophoresis.

Fig. 2A illustrates the hybridization process between the tracer (star) tagged probes (P) and affinity or biotin (B) tagged single stranded RNA analyte sequences and the formation of hybrids (H) between the analytes (A) and the probes (P).

Fig. 2B illustrates the hybridization process between probes (P) with tracer tags (star) simultaneously acting as resolution enabling tags and affinity or biotin (B) tagged double stranded polynucleotide or RNA analyte sequences and the formation of hybrids (H) between the analytes (A) and the probes (P). Probes, which do not match analyte sequences, or which are present in molar excess, remain free in solution.

Fig. 3A depicts the capture of the affinity (B) tagged hybrids (H) to a solid separation aiding tool (SAT) covered with the counterpart of the affinity tag (B).

Fig. 3B depicts the capture of the affinity (B) tagged hybrids (H) to a solid separation aiding tool (SAT) covered with the counterpart of the affinity tag (B). Tracer tagged probe sequences which have not hybridized with an affinity tagged analyte sequence are not captured. Naturally, the separation aiding tools (SAT) bind free affinity tag as well as such affinity tagged analytes to which no probe sequence has hybridized.

Fig. 4 depicts release using elution of the tracer tagged probes (P) from the solid separation aiding tool (SAT)/leaving the affinity tagged analyte sequence (A) with the separation aiding tool (SAT) and tracer tagged probe (P) in solution.

Figs. 5 A-B depict a 16S rRNA approach in microbial ecology.

Fig. 5A depicts a ribosomal RNA gene operon including 16S, 23S and 5S rRNA with the variable regions 1-9 of the 16S rRNA highlighted.

Fig. 5B depicts the structure of 16S rRNA with the variable regions allowing species identification and more or less conserved regions allowing identification of microbial groups.

Fig. 6 depicts a phylogenetic tree of clostridia and related bacteria.

Fig. 7 shows the results, which can be recorded from an electropherogram and from a data file when carrying out the comparative process of the invention according to Example 1. All probes are functional in hybridization with *C. symbiosum* E981051 RNA. Bact and Erec probes have different sizes (18 and 19 bases, respectively) and different mobilities in capillary electrophoresis. The electrophoretic mobility of the Erec-5A probe is different from that of Erec probe due to the addition of A-tail.

Figs. 8 A-B show the result, which can be recorded from an electropherogram and from a data file obtained when carrying out the comparative process of the invention according to Example 2.

Fig. 8A shows the result with the probes Bact and Chis. Chis probe identifies only strain *C. tyrobutyricum* E99908, whereas Bact probe identifies all bacterial strains. Neither probe identifies fungus *Trichoderma reesei.*

Fig. 8B shows the result with the probes Bact and Erec. Erec probe identifies only strain *C. symbiosum* E981051, whereas Bact probe identifies all bacterial strains. Neither probe identifies fungus *Trichoderma reesei.*

Fig. 9 shows the results, which can be recorded from an electropherogram and from a data file obtained when carrying out the quantitative process of the invention according to Example 3. Bact and Chis probe signal intensities correspond to the amount of *C. tyrobutyricum* E908 RNA used for hybridization.

Figs. 10 A-B show the results, which can be recorded from an electropherogram and from a data file when carrying out the qualitative and quantitative process of the invention according to Example 4.

Fig. 10A depicts results obtained when analysing RNA from *C. symbiosum* E1051 with probes Bact and Erec, RNA from *C. tyrobutyricum* E908 with probes Bact and Chis, and microbial population comprising RNA from *C. butyricum* E908, *C. symbiosum* E1051 and *C. lituseburense* E1853 with probes Bact, Chis and Erec. Bact probe identifies all strains whereas Chis identifies only strain E908 and Erec identifies only strain E1051. The level of fluorophores labelling of probes Bact and Chis are equal whereas that of probe Erec is lower. The proportion of RNA from each strain is given as percentage of the total RNA used for the hybridization.

Fig. 10B depicts results obtained when analysing a microbial population comprising *C. tyrobutyricum* E908 and *C. symbiosum* E1051 with probes Bact and Chis. Bact probe identifies both strains whereas Chis identifies only strain E908. The proportion of RNA from each strain is given as percentage of the total RNA used for the hybridization.

Fig. 11 shows a semi-automated performance of the process as a flow sheet.

A Detailed Description of the Invention

The terms used in the present invention have the meaning they usually have in the fields of recombinant DNA technology and nucleic acid hybridization technology. Some terms in the present invention are, however, used in a broader or somewhat different manner. Therefore, some of the terms are defined in more detail below.

Definitions

The term "target population" means a mixture of individual organisms present in a sample comprising different more or less related individual organisms, which can be organized in groups or subpopulations according to their phylogenetic relationship. Examples of such mixed target populations are found in all crude samples that contain or have contained any living or dead organisms including bacteria, fungi yeasts, plants and animals, etc. Environmental studies can be made e.g. from polluted soil samples. Bacterial populations inhabiting the intestines can be the interest of hygienists. The amounts or relative proportions of *Salmonella*, *Shigella* and *E.coli* in a sample indicate the hygienic standards and possible health risks in food industry, restaurants and kitchens. Yeast populations can be checked for the presence of *Saccharomyces, Torulopsis, Candida,* etc. The amounts or relative proportions of *Aspergillus, Penicillium, Trichoderma* and other fungi can be used as an indication of fungal contamination in buildings. Another useful application of the method eventually providing rapid life saving results is the assessment of the effect of certain antibiotics on a sample from a patient suffering from a disease caused by antibiotic resistant bacteria. Even plants and animals including human beings form populations which can be grouped and tested by the method of the present invention.

The organisms may include any unicellular or multicellular organisms with characterized, partially characterized or uncharacterized genomes, which preferably include highly conserved, partially conserved or hypervariable regions, which allow the identification of the organisms and their organization in groups or subpopulations. The

target population can originate from any specimens that contain or have contained living organisms, including microorganisms, plants, animals as well as human beings. The genomes of *E. coli*, *S. cerevisiae* and human beings represent organisms with genomes which at present are more or less fully characterized. The presence of polymorphism is a particularly interesting subject of the present invention.

In the present invention the population is assessed in the form of a polynucleotide mixture isolated from a sample comprising said population. The sample polynucleotide mixture comprises individual polynucleotide sequences and groups thereof, which can be identified with common, more or less conserved probes. The population can be divided in subpopulations, which represent different phylogenetic levels, including groups, genera, species or subspecies. By assessing the amounts or relative proportions of said individual polynucleotide sequences and subgroups thereof, it is possible to evaluate dynamic variation in the amounts and/or relative proportions of organisms or individual polynucleotide sequences taking place in a mixture of polynucleotide sequences or in a target population by taking sequential samples or by comparing samples from different sites or places.

It is to be noted that the polynucleotide sequences in the sample, i.e. the analytes can be of any size. Generally, they are more or less fragmented polynucleotides. In the present invention the reagents or probes used for identification are oligonucleotide sequences, which have approximately the same size. The oligonucleotide probes are rendered distinct with distinguishable sizes by providing them with resolution enabling tags. By definition oligonucleotides comprise from 2-30 base pairs. In the present invention the oligonucleotide probes giving sufficient specificity, preferably comprise at least 10, more preferably at least 15, most preferably about 18-35 base pairs. Principally, there is no upper limit, but it is self-evident that short probes are more cost effective and easier to prepare and handle. The particular problem which is solved by the characteristic feature of the present invention is not the length of the oligonucleotide probes but how to get oligonucleotides sequence of approximately the same size sufficiently distinguishable to enable accurate recording of the results.

The term "pool" means a subset or a library of soluble or solubilizable oligonucleotide probes, i.e. relatively short polynucleotides with approximately the same size. Each pool comprises an optional defined number of oligonucleotide probes. A convenient optional number is, for example, approximately 10 probes. However, the method can be used with as few as two or three probes, but a more convenient number of probes is five or more probes in each pool. Test kits with pools comprising hundreds of soluble probes

can be prepared and used in the quantitative or comparative method of the present invention. Even if it is possible to prepare pools comprising thousands of probes, a preferred upper limit seems to be approximately 300-500 different probes in order to obtain a satisfactory resolution when recording the results. In other words, it must be possible to distinguish the probes from each others by electrophoretic or chromatographic techniques or by mass spectrometry. The pools are said to be "organized" because the contents of each pool are known and are placed in an organized, defined and recognizable manner in their own vessels, which are marked and named to allow their identification. For example when series of pools are prepared on identical microwell plates, each well is characterized not only by its content but also by its place. Thereby identification is accurately enabled.

In the present invention the "pools of oligonucleotide probes" means a set of soluble oligonucleotide sequences, i.e. DNA fragments, which are made from selected oligonucleotide probes, capable of identifying certain groups of organisms having an oligonucleotide sequence, in common, e.g. conserved motifs. Such common oligonucleotide sequences are well known and comprise more or less conserved regions, which can be found especially in ribosomal RNA (rRNA), etc., but they are also present in other nucleotide containing tissues and organelles.

Ribosomes are present in all living cells and are known to comprise proteins and ribosomal RNA (rRNA). Said rRNA in turn comprises alternating conserved and variable regions with nine variable regions found for example in the bacterial 16S rRNA (Fig. 5). The rRNA genes (rDNA) are organized in *rrn* operons, where rDNA genes are separated by hypervariable spacer regions. Most organisms carry several *rrn* operons in their genome and in most cases the intragenomic sequences of the structural rRNA are highly similar. Analysis of rDNA sequences data, especially that of a small subunit rDNA has revealed variable regions in the gene sequences that contain information specific for different phylogenetic levels; groups, genera, species or subspecies (Fig. 6). Thus, sequences unique to certain organisms can be found. This has been utilized to design species and group-specific nucleic acid probes for detection and identification of bacteria and other microorganisms. Such more or less conserved regions or motifs which are more or less common for a multitude of other organisms, enable the individual organisms in a target population to be organized in certain groups or subpopulations and therefore, allow the identification and comparative assessment of variations of individual organisms and subgroups in the target population. DNA and RNA from other sources also comprise more or less variable or conserved regions, which can be used for specific identification of individual organisms or certain subgroups in target populations.

Oligonucleotide probes for other genes and the corresponding messenger RNA (mRNA) can be used to monitor functional properties such as antibiotic resistance in bacteria and gene allele polymorphism.

For accurate recording, the soluble oligonucleotide probes must have sizes which make them distinguishable. In the present invention this is achieved by providing the probes with so called "resolution enabling tags". The oligonucleotide probes of the present invention, which enable identification of related groups of organisms and which are especially useful in the application of the present invention, are generally of approximately the same size. Before use, said oligonucleotide sequences have to be modified and provided with features, which make them distinguishable in a size-based separation, fractionation or recording system. This can be achieved by end-tailing the oligonucleotide sequences with "resolution enabling tags", i.e. residues, including chemical groups or substitutents, providing the oligonucleotide probes with different sizes or masses to electric charge ratios, which enable different mobilities in the fractionation, separation or recording systems used. Preferably, the resolution enabling tags should simultaneously function as affinity, tracer or primer tags. Preferably said tags should have more than one of desired functions.

For example, oligonucleotides probes can be provided with oligonucleotide sequences, including polyA, polyT, polyU, polyC, polyG, mixed polyoligonucleotides, e.g. polyATs, polyGCs or other nucleotide combinations or other oligonucleotide sequences including any mixtures thereof. In addition to being resolution enabling tags, these oligonucleotide sequence can act as affinity tags and primer tags. Tracer tags or labels, e.g. fluorophores of different sizes not only enable detection, they are also useful resolution enabling tags. Amino acids or peptides, which do not disturb the hybridization reaction can be used as resolution enabling tags, but they can also function as affinity tags and tracer tags. There are several strategies reported for the synthesis of peptide oligonucleotide conjugates, which all are readily adaptable for the present invention. In order not to disturb the hybridization it is recommendable to attach the resolution enabling tag only to one end of the probe.

"Tracer tag", means a label or marker, which enables the detection and/or recording of the probe. In the basic embodiment of the present invention the tracer tag is a detectable or recordable marker or label such as a fluorophore. It is to be noted that the tracer tag is preferably placed in one end of the probe. The probe is end-tagged in order to prevent the tracer from disturbing the hybridization reactions between the probe and the analyte. In the present invention the tracer tag can also function as the resolution enabling tag by

providing the probe with a distinguishable mass or size or electric mobility.

The term "tracer tags" means labels or markers, which are visible or otherwise detectable, i.e. directly recordable or which can be made detectable or recordable when contacted with other reagents. Tracer tags, recordable by their electrochemical or magnetic, including mass spectrometric properties, fluorescence, luminescence, infrared absorption, radioactivity or by enzymatic reactions, are especially appropriate. However, it is evident that any other tracer tags not mentioned herein, which tags are easily recordable by automatic means or instruments can be used. It is to be noted that no tracer tag is needed when mass spectrometry or electrophoretic techniques are used for recording, but the approximately same sized oligonucleotide probes have to be provided with other groups enabling resolution by size, mass or mass to electric charge ratio.

Fluorescent dyes such as 2-((iodoacetyl)amino)ethyl)aminonapthylene-1-sulfonic acid) (1,5-IEDANS), fluorescein, Bodipy, FTC, Texas Red, phycoerythrin, rhodamines, carboxytetramethylrhodamine, DAPI, indopyras dyes, Cascade Blue, Oregon Green, eosins, erythrosin, pyridyloxazoles, benzoxadiazoles, aminonapthalenes, pyrenes, maleimides, coumarins, MBD, Lucifer Yellow, Propidium iodide, porhyrins, CY3, CY5, CY9, lanthanides, cryptates, lanthanide chelates, or derivatives or analogues of said tracer molecules are examples of suitable tracer tags. The fluorescent polynucleotide probes are especially useful in automatic or semiautomatic recording of the results combined with continuous flow systems and instruments. Fluorophores with sizes and masses differing to such a degree that they make the oligonucleotide probes distinguishable can be found among those mentioned above. Especially, phosphoramidites such as 6-FAM™, VIC™, NED™, ROX™ and PET™ (all trademarked by Applied Biosystems) can be used to end label oligonucleotides.

In certain embodiments of the present invention very small quantities of the analyte nucleotide has to be identified and a more sensitive test is required. In such cases the probe is provided with a pair of terminal primer sequences or "primer tags", which allow the amplification of the quantitatively recovered probes. Also in this case the probes can further be provided with optional tracer tags, e.g. with fluorophores of different sizes. These primer tags placed in the 3'- and 5'-terminal ends of the probe allow amplification of the probes after a quantitative recovery of the probes hybridizing with the affinity tagged analytes. One of the primer sequences can be quite short, whereas the other can be longer and simultaneously act as an affinity tag and a resolution enabling tag. In this embodiment the probes can be provided with an optional tracer tag during or after the amplification. If mass spectrometry is used for recording, no tracers are needed.

Amino acids and peptides, which do not disturb the hybridization reaction can be attached, preferably end-tagged to the oligonucleotide probes. There are several strategies reported for the synthesis of peptide oligonucleotide conjugates, which all can readily be adapted for the present application. (See e.g. Lönnberg, H. Annu. Rep. Prog. Chem., Sect B 1999, 95, 207-234 and 2001, 97, 177-208). Similar chemical methods for preparing probes of different sizes can be used to link also other organic chemical residues than peptides to the oligonucleotides. Said amino acid or peptide sequences can simultaneously act as "affinity tags" and/or "tracer tags". The amino acid histidine is a useful example. Peptides, including ligands can be used as "affinity tags". Peptides with enzymatic activities can act as "tracer tags". Peptides functioning as antibody-antigen pairs can act as affinity and tracer as well as resolution enabling tags.

The term "analytes" means the polynucleotide sequences, which are obtained from a sample comprising the target population. These polynucleotide sequences may include any nucleotide sequences, (DNA or RNA), including messenger RNA (mRNA), transfer RNA (tRNA), but ribosomal RNA (rRNA) or genes encoding such are especially useful. The target population can be sampled in different sites or places, and at different points of time, e.g. before and after a certain treatment. The polynucleotide sequences in the sample of the target population are isolated by *per se* known methods (Sambrook, J. and Russel, D.; Molecular cloning - A Laboratory Manual, Third Edition (2001)). The sample preparation comprising the analyte polynucleotide sequences can be modified to include a suitable affinity tag.

Preferably the analyte polynucleotides are affinity tagged by a chemical reaction, in which e.g. biotin residues are covalently linked to the polynucleotides or nucleic acid molecules to be studied resulting in modified polynucleotide analytes, i.e. a biotinylated polynucleotide analytes. In order to avoid that steric hindrances disturb the hybridization reaction between the tracer tagged probes and the polynucleotide analytes, the polynucleotide analytes are tagged with the smaller counterpart of the affinity pair, whereas its bigger counterpart is attached to a solid support or separation aiding tool. For analysing the composition of populations as represented by polynucleotide sequences, the affinity-tagged analyte polynucleotide sequences can be polynucleotide sequences of any kind, including total RNA or rRNA or gene preparations. The affinity tag and its counterpart or pair provides a so called affinity-pair, which allows the capture of affinity tagged substances to a solid support, which in this case is called a separation aiding tool.

"Affinity aided solution hybridization" is a well known method wherein the hybridization

reaction between a probe and an analyte nucleotide sequence is allowed to take place without any steric hindrances in a solution. The affinity tag allows the hybrids to be captured on a solid phase, which allows the separation and washing of the collected nucleic acids and thereafter the captured hybrids or probes can be released and measured.

"Affinity tags" applicable also as resolution enabling tags are found among oligonucleotide residues, amino acid residues such as histidine, peptides or sugar residues and also include haptens such as biotin. Some of these tags can also function as tracer tags. For example, oligonucleotide residues can be used as affinity tags, primer tags and resolution enabling tags.

The term "affinity tags" means that the analyte polynucleotides are provided with a label or marker, which has a high affinity to another substance. In other words, the affinity tag is prone to form a strong bond with its counterpart or affinity pair. The strong bonds formed between affinity pairs enable the affinity-pair to act as means for capturing desired substances. A useful affinity pair is for example biotin-avidin or biotin-streptavidin, but other synthetic or non-synthetic "affinity pairs" or binding substances can also be applied. Suitable "affinity pairs" can be found among receptors and ligands, antigens and antibodies as well as among fragments thereof. The preferred "affinity tags" of the present invention include smaller molecules such as biotin, histidine oligonucleotides, haptens, glycans, etc., whereas the preferred counterparts of the "affinity tags" include bigger molecules such as avidin, streptavidin, metal chelates, antibodies, lectins, etc. are used to cover the "separation aiding tool".

The term "separation aiding tool" means preferentially solid supports, such as micro-beads, latex particles, magnetic particles, threads, pegs, sticks, microwells, affinity columns, which are provided with or covered with the counterpart or affinity pair of the "affinity tag". Optionally, the separation aiding tool can include e.g. phase separation or electrophoretic means, which are dependent on the presence of the counterpart of the affinity tag.

The pools of "soluble oligonucleotide probes" are preferably prepared from a more or less characterized library of oligonucleotide sequences using different methods including isolation from nature, synthetic methods, PCR-techniques or recombinant DNA techniques or combinations thereof (Sambrook, J. and Russel, D., Molecular cloning - A Laboratory Manual, Third Edition (2001)). The different oligonucleotide probes capable of demonstrating a specific subgroup or individual, are arranged or placed in pools so that all oligonucleotide probe molecules that represent a certain subpopulation have a

distinct or characteristic size or mass to electric charge ratio, which enable their identification when using capillary or gel electrophoresis or mass spectrometry.

Even if the use of characterized probes are preferred it is possible to prepare probe pools for poorly characterized genomes in the same manner as described in PCT/FI02/00023. and thereafter provide these oligonucleotide probes with resolution enabling tags defined above allowing their separation and recording.

The term "modified oligonucleotide sequences" means that the set of synthetically prepared oligonucleotide probes can conveniently be modified, e.g. the sugar phosphate backbone of the nucleotide sequences can be replaced by peptide bonds or made of so called locked nucleoside analogs. Modified polynucleotides are, for example, peptide nucleic acids (PNAs) described e.g. in WO 96/20212 or locked nucleic acids (LNA), described e.g. in WO 99/14226. Said modified polynucleotide probes can conveniently be applied in the method and test kits of the present invention. They can be copied using genomic DNA or cDNA as models. Often, they have improved properties, including improved stability and they may also have the advantage of being more easy to provide with tracer tags than natural DNA probes.

The "soluble organized pool" comprising "soluble or solubilizable oligonucleotide probes" may be contained in any kind of vessels, which may be totally separate or connected either in a non-fixed or a rigidly fixed manner. In its simplest form, an organized pool comprises one or more vessels, for example test tubes or bottles, which can be connected together in a non-fixed manner for example in a rack for test tubes. A practical example of organized pools placed in vessels, which are connected together in a rigidly fixed manner is provided by the compartments or wells in or on a microtiter plate. As said above the soluble pools are preferably placed in an organized manner, e.g. in the wells on the microtiter plate. The soluble pools are organized in such a way that each pool and each polynucleotide probe in said pool is distinctly identifiable. Microtiter plates with their wells are typical, commercially available embodiments allowing organization and simultaneous handling of many organized pools. Naturally, other tailor-made more convenient organized pools with multiple compartments can be developed and constructed and provided with appropriate marks and instructions for use.

The results are recorded by optional automatic or semiautomatic means or instruments, including electrophoretic or chromatographic techniques as well as mass spectrometry. The whole system can be fully or partly automized.

The General Description of the Invention

The present invention is related to a method, which allows simultaneous, quantitative determination of the amounts or relative proportions of more than one individual polynucleotide or subgroups thereof in mixture of polynucleotide sequences using oligonucleotide probes having approximately the same size. In one embodiment of the present invention the polynucleotide sequence represent selected individual organisms, subgroups, genera, species or strains, which are present in a sample representing a target population of more or less related organisms. Variations in the amounts of subgroups or individual organisms in the population due to inherent causes, such as aging or external stimuli, such as antibiotic treatment, hygienic measures, can be assessed. In another embodiment of the invention variations in the amounts or relative proportions of transcripts of polynucleotide sequences in a single organism can be determined. This allows for example the demonstration of differences in the expression of non-homologous, allelic genes in a chromosome and may explain the reasons for different manifestation of certain diseases, such as cystic fibrosis. It also enables the studies of polymorphism in one organism. The method and test kit are applicable for environmental and population studies. In the basic principles of the method of the present invention comprises a hybridization reaction that is allowed to take place in a solution and the hybrid formed is collected or captured on a solid support provided or covered with the counterpart or affinity pair of an affinity tag. The covering is achieved by chemical means, e.g. by conjugation. Sometimes the affinity between the surface(s) of the solid separation aiding tool and the counterpart of the affinity tag is sufficient to form a stable binding. Tracer-tagged, preferably end-tagged oligonucleotide probes from a previously characterized, partially characterized or uncharacterized pool (library) are contacted with the affinity-tagged analyte polynucleotide sequences obtained from the sample to be analyzed.

One or more soluble pools are provided with preset, but optional numbers, preferably varying between 2-500, more preferably between 5-400, most preferably between 10-300 soluble oligonucleotide or polynucleotide sequences. A prerequisite for the method is that the oligonucleotide probes, which are of approximately the same size, are made distinguishable by attaching or end-tailing the oligonucleotide probes with "resolution enabling tags" which allow their separation or fractionation and enables resolution of the individual oligonucleotide probes in such a manner that an accurate identification and calculation of results can be obtained, e.g. by electrophoretic techniques or mass-spectrometry.

The soluble oligonucleotide probes can be identified without any tracer tags using e.g. mass spectrometry. Alternatively, they can be provided with tracer tags, which in the basic embodiment of the present invention are directly detectable or recordable labels and markers which simultaneously may act as resolution enabling tags. In a more advanced embodiment of the invention allowing a more sensitive assessment, the oligonucleotide probes are provide with a pair of terminal primer tags, which enable an amplification reaction after the quantitative recovery of oligonucleotide probes. During the amplification the probes can be provided with tracer tags using e.g. tracer tagged primers or labeled nucleotides. In this case the resolution enabling tag cannot be a tracer tag. The soluble pools are placed in an organized manner in their own vessels, which can be separate, loosely connected or removable. The organized pools can also be placed in or on a more compact structure, wherein the vessels are more or less rigidly joined together as the wells on a microtiter plate.

In the basic embodiment of the present invention resolution enabling tags, providing the oligonucleotide probes with differences in the size or mass to electric charge ratio are allowed to hybridize with or without tracer or primer tags with the analyte polynucleotide preparation obtained by isolating from the sample containing the target population. The analyte polynucleotide sequences, present in the sampled target population are isolated by *per se* known methods. Generally, the analyte polynucleotides to be determined from target population are ribosomal RNA (rRNA), messenger RNAs (mRNA) or their corresponding genes (DNA). Said analytes are provided with at least one affinity tag, such as biotin, histidine oligonucleotides, haptens or glycans. The analyte polynucleotides are preferably labelled with biotin.

After these reagent preparation steps, the hybridization reaction between the probes and the analytes is allowed to take place. Hybrids are formed in a molecularly accurate quantitative manner between the soluble oligonucleotide probes and the affinity tagged analytes. Because the different oligonucleotide probes present in the pools are known and because there is an excess of each probe as compared to the analytes, it is evident that the hybridization reaction between the analytes and the probes, which results in a hybrid is stochiometrical and the amount of probe recovered corresponds exactly to the amount of analyte polynucleotides present in the sample. Naturally, the analyte sequence need not be a rRNA sequence. It is possible by the present method to quantitate any single stranded sequence as well as any double stranded sequence, after a denaturation step rendering the double stranded analyte single stranded.

As described above by the hybridization in solution DNA:RNA (DNA:DNA) hybrids will form. Generally, the solution hybridization is performed in conditions, which drive the hybridization towards the formation of hybrids, including DNA:DNA, DNA:RNA, RNA:RNA, PNA:DNA, PNA:RNA, LNA:DNA, LNA:RNA etc. The most preferred conditions vary depending upon the oligonucleotide probes, analytes, etc. Thereafter, the hybrids, by the aid of the analyte polynucleotide sequences carrying the affinity tag due to their affinity to their counterpart are collected or captured on the separation aiding tool covered by said counterpart of the affinity tag. Only such oligonucleotide probes which have been able to hybridize with analyte sequences are collected on the separation aiding tool and can be quantitatively recovered, optionally amplified and recorded. The captured and collected hybrids are removed or separated from the hybridization solution and can be washed free from other reagents. The oligonucleotide probes, which have not formed hybrids with the affinity-tagged polynucleotide analytes will remain in the hybridization or wash solutions and accordingly they are removed. The captured and collected hybrids can be washed free from excess probes, including such probes which have not been able to hybridize with an affinity tagged analyte sequence. In such cases, an analyte sequence representing a certain individual organism or subgroup in the target population and corresponding to the oligonucleotide probe has not been present in the sample. The collected oligonucleotide probes, which can be separated or released from the analyte are optionally provided with a tracer tag. In this case the resolution enabling tag cannot be a tracer tag. Redundant affinity tags and affinity tagged analyte sequences, which have not been able to hybridize, because no corresponding probes have been present in the pool are naturally captured on the solid separation aiding tool, but can be separated from the hybrids during the elution and subsequent separation processes. Also such affinity tagged analytes, which do not have a complementary strand among the probes are captured on the separation aiding tool, but they do not disturb the stochiometry of the hybridization process and they do not disturb the consequent analytical steps. They can, for example, be destructed or removed when the probes are isolated or released from the hybrid and/or the separation aiding tool.

Optional separation aiding tools are required in the method of the present invention in order to recover the hybrids formed between the optionally tracer tagged probes and the affinity tagged analytes. The separation aiding tools, which are solid supports, such as microparticles, microbeads, latex particles, magnetic particles, threads, pegs, sticks, microwells and affinity columns are provided or covered with the counterpart(s) or affinity pair(s) of the affinity tags. The separation aiding tool may comprise means for phase separation or electrophoretic means for capturing the counterpart of the affinity tag.

The hybrids recovered on the separation aiding tool are subsequently released from the tool first by eluting, and thereafter by breaking the hydrogen bonds of the hybrids and the optionally tagged individual probes which have been released from the hybrids are isolated, separated by their sizes and recorded with means allowing their quantification. Because each probe represents an analyte polynucleotide sequence in the sample, the amounts or proportional ratios if individual polynucleotides sequences representing individual organisms can be quantitated on a molecular basis. Alternatively, the bonds of the hybrid are first broken and thereafter the solid support and the solution containing the probes are separated from each other by an appropriate method dependent on the separation aid used. Thereafter, e.g. by centrifugation, the probes are separated based on their size and recorded by means allowing their quantification. The purified and isolated probes on the separation aiding tools are eluted with a solution, such as NAOH, NH_4OH or formamide capable of breaking the bonds between the polynucleotide strands.

Consequently, only those oligonucleotide probes which have been able to hybridize to an analyte polynucleotide representing a certain individual organism or subgroup of organism in the target population, i.e. only those oligonucleotide probes, which have a complementary stranded analyte present in the sample are captured by the separating aiding tool and can be recovered for recording.

This means that the automatically or semiautomatically detectable or recordable optionally tracer tagged probes, which are identifiable by their distinct sizes, are captured or recovered and subsequently released or isolated for recording. It is evident for one skilled in the art that the order of performing the steps in certain case can be changed.

If the tracer tag is lacking, the probes can be directly recorded with mass spectrometry. If the tag is a tracer, e.g. a fluorescent substance the probe can also be directly recorded when it has been separated from the analyte polynucleotide, which does not have any tracer tag. The optionally tracer tagged reagent probes are now present in an isolated and free form and their amount corresponds exactly to the amount of analyte nucleic acid previously hybridized to them.

If the tag is a pair of terminal primers, optionally with a tracer tag, the probe can be amplified after separation from the analyte polynucleotide sequence and provided with a tracer tag either during or after the amplification. For example, after an optional number of amplification cycles, the oligonucleotide probe can be provided with a tracer tag and

recorded. Alternatively, the complementary primers can be provided with tracer tags, thereby the probes are provided with tracer tags during the amplification. The amplification allows the recording of subpopulations or polynucleotide sequences present in such minimal amounts that it is under detection limit when other methods are used. In said advanced embodiment of the present invention, which allows a more sensitive assessment of the analyte polynucleotides, the tags on the probes are terminal primer sequences. The terminal primer tagged probes are allowed to hybridize with the affinity tagged analyte polynucleotides in the same way as in the basic embodiment of the present invention. After the stochiometric hybridization reaction, the hybrids are captured on a separation aiding tool and the primer tagged probes are recovered by *per se* known methods. The amount of recovered probes, which exactly correspond to the amount of analyte polynucleotides present in the sample can be amplified an optional number of times by *per se* known PCR-techniques. Thereafter or during the amplification, the probes are optionally provided with tracers and the amount of the probes is recorded. Because the recovery of the primer tagged probe is quantitative and corresponds exactly to the number of analyte molecules and it is known how many times the probes were amplified, i.e. multiplied or copied, it is easy to calculate the amount of analyte in the original sample. This allows a quantitative assessment even of such analyte polynucleotides, which without the amplification would have been under detection limit and thus not recordable. Accordingly, the sensitivity of the method of the present invention can be highly increased. This is a great advantage, if a very sensitive test is needed, for example when the sampled population, e.g. a biopsy sample, contains only a few organisms.

Thus, the affinity selected probe profile can be assessed by sensitive automatic or partly automized, quantitative recording systems, after separating the probes from each other based on their size, e.g. by chromatographic, electrophoretic techniques, including capillary or gel electrophoresis as well as mass spectrometry. The oligonucleotide probe, when rendered recordable by providing it with a distinguishable size or charge and present in a specific pool always corresponds to a specific analyte molecule. Hence, the individual polynucleotides in a mixture of polynucleotides or in a mixed target population can be very accurately deducted.

A comparative quantitative assessment of variations in the amount of various polynucleotides present in cell or tissue sample as a response to inherent changes due to inherent control mechanisms or as a response to external stimuli, including drugs, pathological states requires at least two organized soluble pools, but preferable at least one organized pool for each sample to be tested. Each pool comprise identical

polynucleotide probes, but the organized pools, e.g. each in its own well on a microtiter plate, is optionally provided with a recordable tracer tag. If tracer tags are used it is advantageous to use distinguishable tracers, e.g. fluorophores having different wavelengths of emission. In a preferred embodiment the soluble pools are provided on microtiter plates. Each microtiter plate is otherwise identical, but each has its own specific recordable tracers, which if they are fluorophores preferably emit at different distinguishable wavelengths of emission, which allows simultaneous recording of the variations. It is possible to compare the amounts without tracer tags using mass spectrometry and allowing computer based automatic systems to calculate and compare the recorded results.

The following flow chart of the method describes how to carry out the present invention:

Preparative steps

Step 1 - Preparation of organized pools of soluble oligonucleotide probes having approximately the same sizes

Case 1 - selecting regions from ribosomal RNA

The rDNA fragments are selected to represent more or less conserved or variable regions representing a certain species or group of bacteria or microorganisms. The DNA fragments are provided with resolution enabling tags or tails or labels allowing a good resolution in the size fractionation-stage.

(a) poly A- tailing (See step 2)
(b) tracer labeling (See step 2)
(c) protein- tailing (See step 2)

Case 2 - selecting regions from other sources

The oligonucleotides are selected to represent regions of other genes e.g. antibiotic resistance genes or their corresponding mRNA. Oligonucleotide sequences capable of distinguishing between different alleles of the same gene can also be selected.

Step 2 - End-labelling the DNA probes with a tracer, fluorophores or size providing tail

Preferably, two (or more) sets of the DNA with distinguishable dyes are prepared. This allows simultaneous comparative studies of variations in polynucleotide amounts, particularly rRNA amounts due to shifts in populations or internal mechanisms, e.g. pathological stages or due to external stimuli, such as drugs. Steps 1 and 2 are preparative and the bases for the commercially valuable test kits. The DNA pools can be

made in large quantities for a large number of experiments. Accordingly, there should not be any need to repeat this rather tedious phase frequently.

Analytical steps

Step 1 Preparation of a single stranded polynucleotide analyte

Nucleic acid is isolated from the mixed population pool by *per se* known methods. The isolation of RNA from the cells is used during appropriate experimental conditions using *per se* known methods (Sambrook, J. and Russel, D., Molecular cloning - A Laboratory Manual, Third Edition (2001)). If the polynucleotide analyte is double stranded the analyte has to be denaturated in order to provide the single stranded sequences required in the method of the present invention.

Step 2 Preparation of affinity tagged analytes

The isolated mRNA is affinity tagged, for example biotinylated using a chemical, non-enzymatic process. The photoactivated reagent photobiotin is convenient for this purpose and it is commercially available. As the RNA will not be transcribed to cDNA or otherwise enzymatically modified for labelling, the RNA can be prepared and kept in strong detergents such as SDS. RNAses are inhibited by SDS so it is easy to isolate intact RNA. However, fragmentation is not a problem if not too heavy. The size of the RNA fragments will not affect the capturing capacity.

Step 3 - Solution hybridization

Contact each of the soluble tracer tagged probe (DNA) pools with an aliquot of the affinity tagged analyte (RNA) preparation. Allow the hybridization to take place in the free solution in the small volume provided in respective pool compartment. This gives a fast and quantitative reaction.

Step 4 - Separation step

Add microbeads or another separation aiding tool carrying the affinity pair, e.g. avidin to capture the RNA molecules. Wash to get rid of free DNA.

Step 5 - Recovering stage

Elute with a solution which breaks the DNA:RNA hybrid such as formamide or NaOH. If necessary, precipitate and wash the single-stranded DNA. Take up the single stranded DNA in an electrophoresis buffer. It is preferable that such conditions are used that electrophoresis of the eluate can be carried out directly and the different probes recorded simultaneously.

Step 6 - Recording of results

Determine the size and amount of DNA eluted from DNA:RNA hybrids by capillary or gel electrophoresis. Mass spectrometry can possibly be used as well. Differences in two RNA preparations are easily observed by hybridizing to DNA fragments labeled with different dyes and mixing the DNAs prior to electrophoresis.

Step 7 - Interpretation of the results

In case 1 in the composition of the population becomes directly determined in the respect of subpopulations for which probes were included in the pool. Likewise, in case 2 the presence of certain functional properties (presence or expression of genes) in an individual organism or a population becomes directly determined.

Step 8 - Optional amplification

If a very sensitive assay is needed the reagent polynucleotide sequences, i.e. the tracer-tagged probes eluted from the separation aiding tool can be amplified by PCR after the quantitative selection step. If this approach is used the reagent polynucleotide sequences, i.e. the probes should be modified to contain a common terminal sequence allowing amplification of all the probes in the same pool with the same PCR primer pair, provided with a tracer tag.

When the probes are provided with tags or tails allowing their separation by size or mobility, they can be recorded based on their masses using mass-spectrometry. In this case, no tracer tags are required and further improvement of the method is enabled. By omitting the use of tracer tags, the method can be simplified and the need of expensive recordable labels can be avoided. Otherwise, the method fully corresponds to the method as described above and comprises the following consecutive steps:

(a) providing, one or more organized pools with a preset optional number of soluble probe oligonucleotide sequences with distinct sizes allowing their identification or recording, said pools being placed in an organized manner in their own vessels which are separate or joined together;

(b) isolating the analyte polynucleotide sequences present in a cell or tissue sample of the target organism and providing said analytes with at least one affinity tag;

(c) allowing a hybridization reaction to take place between the soluble probes from the step (a) and the analyte from step (b) leading to formation of soluble probe:affinity tagged analyte-hybrids;

(d) isolating the probe:analyte-hybrids formed in step (c) by capturing said hybrid on a separation aiding tool provided with the affinity pair of the affinity tag of the analyte;

(e) recovering the probe from the separation aiding tool; and

(f) recording the size and amount of probe with electrophoretic techniques or mass spectrometry.

Test kits

The present invention is also related to a test kit. The test kit comprises one or more soluble organized pools with a preset optional number of soluble oligonucleotide probes, which hybridize with more or less conserved or variable regions which are common for the whole population or specific for a certain subgroup of organisms. Alternatively, the test kit comprises probes which hybridize with specific genes encoding for certain functions and their corresponding mRNA such as those of antibiotic resistance. The oligonucleotide probes are optionally provided with tags, either tracer tags or a pair of terminal primer tag sequences. Preferably, the tracer tags are end-labelled detectable tracer tags, such as fluorophores, providing different sizes to the oligonucleotide probes.

The test kit comprises soluble organized pools, each pool having more than one, preferably more than ten, most preferably about hundred or more probes. The pools are preferably placed in an organized manner in their own vessels, e.g. test tubes, bottles or in the wells or compartments of a microtiter plate. Even if the test kit for performing the present quantitative determination preferably is a microtiter plate or a corresponding tailor-made structure, the test kit can be an optional number of test tubes, bottles, etc., which can be organized in more or less fixed arrangements, including racks and/or other rigid structures. The test kits can be customized or tailor-made and provided with appropriate marks and instructions for use.

The pools of soluble polynucleotide probes for the test kits can be prepared from fragments of DNA. They can be synthetic oligonucleotides and modified DNAs. When the test kit is prepared for studying characterized genomes, the pools of the test kit preferably comprise at least one polynucleotide fragment (probe) from each gene to be studied in the genome. Also when uncharacterized genomes are to be studied, the pools can advantageously be prepared in larger quantities, commercial production is in no way excluded, for more general or more specific studies.

If the reagent oligonucleotide probes are derived from a characterized genome each probe molecule is known to correspond to a given gene, and each probe is specifically identified by its size and pool. The variations of amounts or relative proportions of organisms or subgroups thereof in a certain mixed population can thus directly be compared and automatically calculated from the automatically recorded results. If the reagent polynucleotide probes are poorly characterized, they are for instance derived

from an organism, the genome of which is not sequenced, valuable results can still be obtained.

A preferred embodiment of the test kit can be prepared on a microtiter plate. In such a practical embodiment of the invention, pools with DNA fragments from known or unknown sequences of yeast, clostridia, bacteria causing food poisoning, etc. can be used for preparing the test kits. If each pool comprises e.g. approximately 10-100 probes or fragments it gives a sufficiently good resolution. If each probe in the pool represents a given bacterial species, probes for thousands of species can be placed on a single microtiter plate and there is still place for a number of controls. The captured DNA probes are identified partly by the pool or microtiter well to which it belongs, and partly by their size.

The optional recordable tracer tag is advantageously selected from a group of tracers detectable by fluorescence, infrared absorption, electromagnetic properties, radioactivity and enzymatic activity. The preferred tracer tag recordable by its fluorescence is a fluorochrome or a fluorophore. Mass spectroscopy is another preferred mode, which allows recording and quantification without any tracer tags. Even if tracer tags are preferred embodiments they are not essential for the method of the present invention, the only prerequisite for the test kit of the present invention is that the probes in the soluble organized pools have distinct sizes. They are optionally tagged, either with tracer tags or terminal primer tags. Accordingly, a working test kit is provided by an organized pool of terminal primer tagged probes even if no tracer is provided.

The test kit of the present invention in its simplest form is an organized pool of soluble tagged probes with distinct sizes. It is to be noted that said test kit is complete as such but can be complemented with optional tracers, affinity pairs and/or separation aiding tools. However, said auxiliary reagents are no prerequisite. Said auxiliary reagents and means for performing the method of the invention are available even commercially from several other sources. Thus, the method and test kit of the present invention can be tailor-made for the specific needs of the end-user, especially they should be applicable for automatic or semiautomatic handling.

The mode of test kit manufacturing, which accordingly need not include immobilization steps, allows for easy adaptation of tailor-made tests, directing the attention to certain subsets or subpopulations of organisms in a given population. The test kit may comprise an optional affinity tag for labelling the polynucleotides in the cell or tissue sample and optional separation aiding tool provided or covered with a counterpart of the affinity tag

for labelling the analyte. The optional affinity pairs providing the affinity tags for the analytes and the counterparts for the separation aiding tools include, but are not limited to, for example, biotin and avidin or streptavidin, histidine and metal chelates, haptens and antibodies or glycans and lectins.

The optional separation aiding tool, which can be incorporated into the test kit or can be provided separately, is selected from a group of solid supports consisting of microparticles, microbeads, latex particles, magnetic particles, threads, pegs, sticks, microwells or affinity columns. The separation aiding tool may include means for phase separation or electrophoretic means for capturing the counterpart of the affinity tag.

For the comparative assessment of variations of the amounts or relative proportions of individual polynucleotide sequences or subgroups thereof in a mixture of polynucleotide sequences, organized pools with identical sets of probes can be provided. In this case, each organized pool or test kit is optionally provided with optionally different or distinguishable tracer tags, which tags are distinguishable based on their sizes or mobilities and preferably emit at different emission lengths.

If the tags are terminal primer tags simultaneously acting as resolution enabling tags the test kits are identical, but after the amplification the recovered and/or amplified probes can optionally be provided with distinguishable tracer tags. Alternatively, the complementary primer pair can be provided with a tracer tag, allowing tracer tagging during amplification. These auxiliary reagents can optionally be incorporated in the test kit or provided from other commercial or non-commercial sources. In order to enable simple comparative assessment of variations, in polynucleotide amounts in a sample, it is convenient to prepare test kits provided with different and distinguishable tracer tag emitting at different emission lengths and which can be recorded with automatic or semi-automatic instruments.

Test kits for comparative quantitative assessment of variations in the amounts of various individual polynucleotides or organisms or subgroups thereof in a mixture of polynucleotides or a target population as a response to inherent changes or external stimuli, including antibiotics, pathological states, epidemiologic conditions, conveniently comprise at least two solid supports or microtiter plates. Each solid support or microtiter plate is provided with identical pools of polynucleotide probes, optionally provided with the tracer tags. Each solid support or microtiter plate should optionally be provided with its own distinguishable tracer tag, which allows simultaneous recording of cell or tissue samples obtained at different times, for example before or after drug treatment.

Population profiling, i.e. analysing the differences in two or more analyte polynucleotide preparations, are easily recordable by hybridizing the analyte samples to reagent polynucleotide probes end-labelled with different, distinguishable and automatically recordable tracer tags. After the hybridization step the different samples can optionally be mixed and their differences directly observed by measuring the ratio of the tracer tags to each other in each peak. The test kit can also be provided with at least one pair of primers for amplifying the tracer tagged probes obtained in the last step, for increasing the sensitivity of the test.

The method of the present invention is useful for quantitative and comparative assessment of variations in the amounts of certain organism and subgroups thereof in a sample of a selected mixed population.

The human gastrointestinal tract is probably the most complex microbial ecosystem described and it has been estimated that at least 400 bacterial species reside in the human large intestine. In order to study this extremely complex ecosystem convenient high-throughput analytical tools such as the described invention are needed. The present invention allows simultaneous screening of the presence of numerous bacterial groups and species and their relative quantitation in gastrointestinal samples. For example in functional food studies a particular interest is to follow changes in the intestinal microbial populations. Bifidobacteria and lactobacilli belong to the indigenous microbial population of the human intestine and they are considered to be the marker organisms of well-balanced gut microbiota. Bifibacteria and lactobacteria often monitored in nutritional interventions. Genus- or group-specific probes as well as many species-specific probes are available for bifidobacteria and lactobacilli and thus, the described invention is readily adaptable for the detection of these bacteria. Another important group of intestinal bacteria are clostridia, some of which are potentially pathogenic. The enumeration of clostridia is troublesome due to inadequate selective media but the described invention provides a culture-independent approach for qualitative and quantitative monitoring of clostridia as well as other microbial groups.

The described invention can also be utilized in clinical microbiology e.g. in assessing the efficacy of antibiotic treatment on bacterial populations. In order to find the correct antibiotic treatment in urgent situations with infections caused by antibiotic resistant bacteria rapid screening methods are especially valuable.

In drinking-water supply and food and feed production hygienic measures and good control are required. Based on the described invention test kits for controlling the

microbiological quality of drinking water and food products can be designed. In food industry reliable tests for pathogenic microbes such as *Salmonella, Listeria, Bacillus* and *E. coli* take priority but also tests for non-pathogenic food spoilage microbes such as lactobacilli and yeasts are often needed.

Another field of application for the invention are test kits for detecting fungi which can grow in building structures and thereby cause serious health problems for humans by releasing toxins and spores to indoor air. Microbes can also cause damage to buildings and historically important artifacts such as ancient wall paintings, sculptures etc. Appropriate test kits can be designed for the identification of causative microbes are and monitoring the effectiveness of control measures.

In microbial ecology culture-independent monitoring methods are essential, because the laboratory growth conditions often fail to mimic the natural environments of microbes and consequently, only a fraction of microbes in environmental samples can be recovered on laboratory media. Different tests can be designed for monitoring uncultured microbial populations in different soil and water samples, which allow the evaluation of the effects of pollution, agriculture and other human actions on natural ecosystems and the efficacy of corrective measures environmental damages such as oil spillage. In fundamental ecological research the monitoring of natural seasonal variations in the microbial ecosystems and the comparison of similar ecosystems in different geographical locations is interesting.

Test kits can comprise of oligonucleotide probes which can discriminate between certain alleles of genes. Such kits can be used for population studies to study the distribution of certain alleles of genes, for example. Likewise oligonucleotides which recognize point mutations in various genes can be used in the kits.

In addition to the applications listed above the method and test kits can be used for evolutionary studies and to evaluate relationship. In archeology it can be used to study the causes of degradation of ancient wall paintings and statues and other artifacts by microbes and monitoring of the effect of preventive measures.

The method and test kits can be used for detection of point mutations with potentially detrimental effects on the health of humans and animals and for population studies including distribution of certain alleles of genes in the population

The test kit of the present invention in its simplest and cheapest form is otherwise the

same as the test kits described above and comprises one or more organized pools with a preset optional number of soluble polynucleotide sequence probes provided with distinct sizes allowing their identification and recording with mass-spectrometry. The probes can be provided with terminal primer tags in order to allow amplification before the quantitative measurement with mass-spectrographic or spectrometric means. Said pools of unlabeled probes are placed in an organized manner in their own vessels, which are separate or joined together.

The test kit including the reagents of the present invention are preferably applicable for carrying out automatic or semi-automated processes, an example of which is shown as a flow sheet in Figure 11. The process can be interrupted and the reagents transferred to other solid supports if the automatic devices are not quite compatible. The first steps are advantageously carried out in an automated pipetting station, wherein the biotinylated sample RNA is pipetted into each pool containing the distinctly sized probes in their pools. Thereafter, the test kit can be dried using a lyophilisator. The drying is made to eliminate the influence of any differences in volumes. The optional lyophilization allows the work to be stopped until it is convenient to continue the work.

The work is recontinued by adding an appropriate hybridization buffer to the pools in an automated pipetting station. The plate is sealed with appropriate means, e.g. a film or a foil in order to avoid evaporation in the subsequent step. When the test kit has been provided with an appropriate heat sealer it is positioned into an automated thermal block, where the temperature can be up- or downregulated as required to enable the denaturation and hybridization of the probes. After hybridization the solution containing the probe:analyte-hybrids are placed in a magnetic particle processor in order to carry out the affinity capture, washing and elution steps by moving steptavidin/avidin coated magnetic beads from step to step e.g. on a KingFisher plate according to a programmed protocol. The eluates can optionally be transferred into a new plate if the automated stations use different types of microtiter plates. The wells can be rinsed with elution buffer for quantitative transfer and then the combined solutions are evaporated in a lyophilisator, which enables preservation of the samples and making the recording at a more convenient time. In other words, the process can easily be adapted for different time schedules and protocols for performing the determination. The probe fragments, size standard and concentration standards, are either directly or after a convenient step, automatically injected into an automatic analyser. The intensities of labels attached to the probe fragments are determined as areas. The areas of the concentration standards, with known amounts, are then used to determine the absolute amounts each probe fragment.

The experimental design and the general principles of the present invention are described in more detail using bacterial strains available in the laboratory of the inventors and synthetic oligonucleotides. The strains and oligonucleotides are used for illustrative purposes only. The invention is in no way limited to said strains and oligonucleotides. The principles of the invention can be checked by replacing the construct used in the examples by any other strains or oligonucleotides, which are available in abundance. Those skilled in the art can easily apply the principles of the invention in different applications.

Example 1
Mobility of probes in electrical field and modification of probes

Total RNA was extracted from *Clostridium symbiosum* strain VTT E-981051^T (henceforward E1051) and hybridized with two 16S rRNA targeted probes Bact (Amann R. I., et al., Appl. Environ. Microbiol. 56:1919-1925, 1990) and Erec (Franks, A. H., et al., Appl. Environ. Microbiol. 64:3336-3345, 1998). Probe Bact is specific for bacteria (previously eubacteria) (Amann *et al.*, 1990), whereas probe Erec is specific for bacteria belonging to the group of *Clostridium coccoides - Eubacterium rectale* (Franks, A. H., et al., Appl. Environ. Microbiol. 64:3336-3345, 1998). The species *Clostridium symbiosum* belongs to the *Clostridium coccoides - Eubacterium rectale*-group and thus its rRNA/rDNA is recognized by both probes Bact and Erec. In addition, Erec-5A - a modified version of the probe Erec with an attached 5A-tail (five additional adenosins) - was used in the model experiment. The experiment followed the steps set forth below:

Preparative steps:
RNase free disposable microcentrifuge tubes, pipette tips, reagents etc. were used in the preparative and analytical steps whenever necessary.

Step 1 - Probes
16S rRNA targeted oligonucleotide probes
5' GCTGCCTCCCGTAGGAGT 3' (SEQ ID NO:1),
5' GCTTCTTAGTCARGTACCG 3' (SEQ ID NO:2) and
5' GCTTCTTAGTCARGTACCGAAAAA 3' (SEQ ID NO:3),
wherein R=A/G are listed in Table 1. and labelled with 6-FAM fluorophore in the 5 end were purchased from Applied Biosystems:

Table 1. Probes

Probe name	Sequence	Length (nucleotides)	Reference
Bact	SEQ ID NO:1	18	Amann et al., 1990
Erec	SEQ ID NO:2	19	Franks et al., 1998
Erec-5A	SEQ ID NO:3	24	buu

Step 2 - Preparation of analyte

Clostridium symbiosum E1051 was grown as a pure culture in reinforced clostridial broth (Difco) in anaerobic conditions at 37 °C. Total RNA from E1051 was extracted according to Zoetendal, E. G., et al., Appl. Environ. Microbiol. 64:3854-3859 (1998).

Analytical steps:

Step 1 - Affinity tagging analyte sequences

RNA was affinity tagged with PHOROPROBE^R Biotin SP-1000 according to the manufacturer's (VECTOR Laboratories) instructions. Subsequently, the biotinylated RNA was purified from free biotin with RNeasy mini kit by applying the protocol for RNA clean-up according to the manufacturer's (Qiagen) instructions.

Step 2 - Solution hybridization

An aliquot of the RNA sample (102 ng) was mixed with oligonucleotide probe (1 pmol) in hybridization solution with final concentration of 5 x SSC (0.75 M NaCl - 75 mM sodium-citrate, pH 7.0), 0.1 % (w/v) SDS and 1 x Denhardt s (0.02 % (w/v) Ficoll, 0.02 % (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin). The volume of the hybridization mixture was 20 μl. The reaction mixture was incubated at 70 °C for 2 min and then at 40 °C for 30 min.

Step 3 - Affinity capture, washes and elution

Following the hybridization, KingFisher magnetic particle processor (ThermoLabsystems) was used to perform affinity capture, washing and elution steps by moving streptavidin coated magnetic beads from step to step on a KingFisher microtiter plate according to a programmed protocol. Solutions for each step were pipetted beforehand to specified wells in the microtiter plates and the procedure was carried out in room temperature.

The hybridization reactions were transferred into specified wells in the KingFisher plate(s). In order to adjust the NaCl concentration suitable for the affinity capture (1M)

and to transfer the hybridization mixture quantitatively into the KingFisher wells the hybridization tubes/wells were rinsed with 40 µl rinsing solution and the rinsing solution was subsequently added to the same KingFisher wells with the hybridization mixtures. The rinsing solution consist of one part of 2M NaCl-10 mM Tris-HCl (pH 7.5) -1mM EDTA and 2.33 parts of hybridization solution (see Step 2).

Biotinylated RNA and RNA-oligonucleotide-hybrids were collected on streptavidin coated magnetic particles Dynabeads^{R} M-280 (50 µg, Dynal A.S., Norway) for 30 min. Following capturing the particles were washed three times with 150 µl 1x SSC (0.15 M NaCl - 15 mM sodium-citrate, pH 7.0) - 0.1 % SDS and twice with 150 µl of water (deionized, ultrafiltrated, RNase free) and the probes were eluted with 30 µl of formamide. Subsequently, the formamide was evaporated in a lyophilisator and the probes resuspended in 10 µl of water.

Step 4 - Identification of eluted probes

The eluted probes were analysed by using ABI310 capillary electrophoresis equipment (Applied Biosystems). The eluted probes were identified based on their migration behaviour in the capillary electrophoresis. Beforehand, free probes were run in the same equipment in same running conditions and their migration behaviour determined. In order to facilitate the comparison of individual runs (i.e. samples) size standard was added to the samples. The result was read from the electropherogram and from the data file as shown in Fig. 7.

As seen in Fig. 7 oligonucleotide probes differing in size only by one nucleotide can be separated as individual peaks in capillary electrophoresis and the addition of 5A-tail to Erec probe significantly altered its migration behaviour in capillary electrophoresis. Despite the modification of probe Erec by the attachment of 5A-tail it recognized the target RNA from strain E1051.

Example 2

Specificity of the probes in a protocol of the invention

The specificity of two probes Chis and Erec (Franks, A. H., et al., Appl. Environ. Microbiol. 64:3336-3345, 1998) in the specified reaction conditions was ensured by hybridizing the probes with a number of bacterial strains. Probe Bact was used as an internal control in hybridization to ensure the integrity of bacterial rRNA. Chis is specific for bacteria belonging to the *Clostridium histolyticum* group (Franks, A. H., et al., Appl. Environ. Microbiol. 64:3336-3345, 1998). The probes Bact and Erec were previously described in example 1. The experiment followed the steps set forth below:

Preparative steps:

Step 1- Probes

Chis 5' TTATGCGGTATTAATCTRCCTTT 3' (SEQ ID NO:4), wherein R=C/T; Franks, A. H., et al., Appl. Environ. Microbiol. 64:3336-3345, 1998) labelled with 6-FAM™ in the 5 -end was purchased from Applied Biosystems. The probes Bact and Erec were previously described in preparative step 1 in example 1.

Step 2 - Preparation of analytes

Pure culture of different microorganisms form VTT Culture Collection (Table 2) were grown in adequate nutrient medium and total RNA from bacteria was extracted as described by Zoetendal, E. G., et al., Appl. Environ. Microbiol. 64:3854-3859 (1998) or with RNeasy mini kit by applying the protocol for the isolation of total RNA from bacteria according to the manufacturer s (Qiagen) instructions. Total RNA from *Trichoderma reesei* was extracted by using the TRIzol® Reagent method (Life Technologies; Gibco BRL).

Table 2. Test organisms

Species	Strain Alternative Codes		Target to probe		
	VTT	International Culture Collection	Bact	Erec	Chis
Clostridium acetobutylicum	E-00022^T	ATCC 824	+	-	+
Clostridium tyrobutyricum	E-99908	DSM663	+	-	+
Clostridium symbiosum	E-981051^T	ATCC 14940	+	+	-
Eubacterum rectale	E-022088	ATCC 33656	+	+	-
Clostridium leptum	E-021850^T	DSM 753^T	+	-	-
Clostridium lituseburense	E-021853^T	DSM 797^T	+	-	-
Trichoderma reesei	D-74075	ATCC 26921	-	-	-

Analytical steps:

Step 1 - Affinity tagging analyte sequences

RNA was affinity tagged with biotin as described in analytical step 1 in example 1. Following biotinylation, the biotinylated RNA was purified from free biotin according to the protocol provided by VECTOR Laboratories or with RNeasy mini kit by applying the protocol for RNA clean-up according to the manufacturer s (Qiagen) instructions.

Step 2 - Solution hybridization

An aliquot of the RNA sample (50 to 80 ng) was mixed with hybridization solution (see analytical step 2 in example 1) containing oligonucleotide probes Bact and Chis or Bact and Erec (1 pmol each). The final volume of the hybridization mixture was 20 μl. The reaction mixture was incubated at 70 ^{o}C for 2 min and then at 50 ^{o}C for 30 min.

Step 3 - Affinity capture, washes and elution

Affinity capture, washes and elution were performed by using the KingFisher magnetic particle processor (ThermoLabsystems) as described in analytical step 1 in example 1.

Step 4 - Identification of eluted probes

The eluted probes were analysed by using ABI310 capillary electrophoresis equipment (Applied Biosystems) as described in analytical step 4 in example 1.

As seen in Fig. 8 oligonucleotide probes Chis and Erec showed the expected specificity (Table 2) in the specified hybridization conditions and gave signal only with strains that belong to their target group. The probes showed expected specificity also with strains E-00022^{T} and E-022088 which are not included in Fig. 8. Further, probe Bact also showed desired specificity and did not produce a signal with *Trichoderma reesei* RNA. RNA from strain E-021850 was partially degraded but still gave a signal with probe Bact showing that the method can be used also to analyse RNA that has been shared for example during the preparative steps. The specified hybridization conditions were the same with all three probes and hence, these probes can be used as a pool of probes.

Example 3

Quantitative evaluation

Total RNA was extracted from *Clostridium tyrobutyricum* VTT E-99908 (henceforward E908) and different amounts of RNA (0.01 - 10 ng) were hybridized with probes Bact and Chis. The experiment followed the steps set forth below:

Preparative steps:

Step 1- Probes

Probes Bact and Chis previously described in preparative step 1 in example 1 and preparative step 1 in example 2 were used in the experiment.

Step 2 - Preparation of analytes

E908 was grown as a pure culture in reinforced clostridial broth (Difco) in anaerobic conditions at 37 °C and total RNA was extracted with RNeasy mini kit by applying the protocol for the isolation of total RNA from bacteria according to the manufacturer's (Qiagen) instructions.

Analytical steps:

Step 1 - Affinity tagging analyte sequences

RNA was affinity tagged with biotin as described in analytical step 1 in example 1. Following biotinylation, the biotinylated RNA was purified from free biotin according to the protocol provided by VECTOR Laboratories.

Step 2 - Solution hybridization

Total RNA extract from E908 was adequately diluted and an aliquot of the RNA sample (0.01; 0.05; 0.1; 0.5; 1.0 and 10.0 ng) was mixed with hybridization solution (see analytical step 2 in example 1) containing oligonucleotide probes Bact and Chis (1 pmol each). The final volume of the hybridization mixture was 20 μl. The reaction mixture was incubated at 70 °C for 2 min and then at 50 °C for 30 min.

Step 3 - Affinity capture, washes and elution

Affinity capture, washes and elution were performed by using the KingFisher magnetic particle processor (ThermoLabsystems) as described in analytical step 1 in example 1.

Step 4 - Identification of eluted probes

The eluted probes were analysed by using ABI310 capillary electrophoresis equipment (Applied Biosystems) as described in analytical step 4 in example 1.

As seen in Fig. 9 the probe signal intensity (peak height and area) correlates well with the amount of RNA used in the hybridization. Both probes have target sites within 16S rRNA molecule but in different regions of the molecule. Probes Bact and Chis have equal level of fluorophore labeling and hence, the signal intensity from the probes are comparable.

Example 4

Analysis of microbial populations

Total RNA was extracted from strains E1051, E908 and *Clostridium lituseburense* VTT E-021853 (henceforward E1853). Different amounts of RNA from these three strains were mixed and hybridized with a pool of probes consisting of probes Bact, Erec and Chis. The experiment followed the steps set forth below:

Preparative steps:

Step 1- Probes

Probes Bact, Erec and Chis previously described in preparative step 1 in example 1 and preparative step 1 in example 2 were used in the experiment.

Step 2 - Preparation of analytes

Total RNA from pure cultures of E1051, E908 and E1853 was extracted as described in preparative step 2 in example 2.

Analytical steps:

Step 1 - Affinity tagging analyte sequences

RNA was affinity tagged with biotin as described in analytical step 1 in example 1. Following biotinylation, the biotinylated RNA was purified from free biotin as described in analytical step 1 in example 2.

Step 2 - Solution hybridization

Specified amounts of RNA from different bacteria (Table 3) were mixed and hybridization solution (see analytical step 2 in example 1) containing oligonucleotide probes Bact, Chis and Erec (1 pmol each) was added. The final volume of the hybridization mixture was 20 μl. The reaction mixture was incubated at 70 ^{o}C for 2 min and then at 50 ^{o}C for 30 min.

Table 3. Hybridization experiments carried out in Example 4.

Hybridization reaction	Probe pool	% RNA of total RNA		
		E1051	E908	E1853
I	Bact, Erec, Chis	100	-	-
II	Bact, Erec, Chis	-	100	-
III	Bact, Erec, Chis	20	20	60
IV	Bact, Chis	50	50	-
V	Bact, Chis	80	20	-

Step 3 - Affinity capture, washes and elution

Affinity capture, washes and elution were performed by using the KingFisher magnetic particle processor (ThermoLabsystems) as described in analytical step 1 in example 1.

Step 4 - Identification of eluted probes

The eluted probes were analysed by using ABI310 capillary electrophoresis equipment (Applied Biosystems) as described in analytical step 4 in example 1.

As seen in Fig. 10A from the signal from probe Erec is lower than the signal from probe Bact when analysing RNA from *C. symbiosum* E1051. This is due to the lower level of fluorophore labelling of probe Erec as compared to probe Bact. The level of fluorophore labelling of probes Bact and Chis are equal and hence, the signal intensity from probes Bact and Chis are equal when analysing RNA from *C. tyrobutyricum* E908. Thus, the level of probe labeling can be used to adjust the detection limit to an adequate level. In qualitative analysis of microbial population comprising of RNA from *C. tyrobutyricum* E908, *C. symbiosum* E1051 and *C. lituseburense* E1853 signal all probes Bact, Chis and Erec gave a signal, as expected. Bact probe identifies all strains whereas Chis identifies only strain E908 and Erec identifies only strain E1051.

As seen in Fig 10B probes Bact and Chis which have equal level of fluorophore labeling can be used to quantify the relative proportion of bacteria belonging to the *C. histolyticum* group (*C. tyrobutyricum* E908) in a mixed bacterial population (*C. tyrobutyricum* E908 and *C. symbiosum* E1051). Bact probe identifies both strains whereas Chis identifies only strain E908.

Claims

1. A method for determination of the amounts or relative proportions of more than one individual polynucleotide sequence or subgroups thereof in a polynucleotide mixture using a quantitative affinity aided solution hybridization comprising the consecutive steps of:
(a) providing, one or more organized pools with a preset optional number of soluble polynucleotide probes, which are distinguishable in order to provide resolution for recording of results, each pool being placed in an organized manner in its own vessel;
(b) providing analyte polynucleotide sequences isolated from a sample comprising a mixture of target polynucleotide sequences with at least one affinity tag;
(c) allowing a hybridization reaction to take place between the soluble oligonucleotide probes from step (a) and the analyte polynucleotide sequences from step (b) leading to formation of soluble hybrids;
(d) recovering the hybrids formed in step (c) by capturing said hybrids on a separation aiding tool provided with the affinity pair of the affinity tag of the analyte;
(e) releasing the probes from the hybrids and separation aiding tool; and
(f) recording the amount of individual polynucleotide probes, **characterized** in that said polynucleotide probes are oligonucleotide probes, which have approximately the same size and/or mass and are made distinguishable by providing said oligonucleotide probes with one or more resolution enabling tags capable of changing the size or mass to electric charge ratio and thereby providing the oligonucleotide probes with different mobilities in the fractionation, separation or recording systems without disturbing the hybridization or capturing reaction.

2. The method according to claim 1, **characterized** in that for the determination of dynamic variations in the amounts or relative proportions of polynucleotide transcripts or their subgroups in an individual organism, the soluble oligonucleotide probes are designed from species or group-specific oligonucleotide sequences hybridizing with selected more or less conserved or hypervariable regions from intragenomic sequences specific for subgroups, species, subspecies of transcripts expressed in the organism.

3. The method according to claim 2, **characterized** in that the polynucleotide analytes isolated from the sample comprising the mixed target population comprise messenger RNA (mRNA).

4. The method according to claim 1, **characterized** in that for the determination of dynamic variations in the amounts or relative proportions of polynucleotide sequences

representing individual organisms or subpopulations thereof in a target population, the soluble oligonucleotide probes are designed from species or group-specific oligonucleotide sequences hybridizing with selected more or less conserved or hypervariable region from intragenomic sequences specific for and/or representing different phylogenetic levels allowing the identification of subgroups, species, subspecies within the mixed target population.

5. The method according to claim 4, **characterized** in that the polynucleotide analytes isolated from the sample comprising the mixed target population comprise ribosomal RNA or DNA.

6. The method according to claim 1, **characterized** in that the resolution enabling tag may simultaneously act as a tracer, affinity and/or primer tag.

7. The method according to claim 4, **characterized** in that the resolution enabling tag is selected from a group consisting of oligonucleotide residues, amino acid residues, peptides, sugar residues, haptens and ligands.

8. The method according to claim 4, **characterized** in that the resolution enabling tag which additionally may act as an affinity and/or primer tag is an oligonucleotide residue.

9. The method according to claim 4, **characterized** in that the resolution enabling tag which additionally may act as an affinity tag and/or a tracer tag is an amino acid or a peptide.

10. The method according to claim 4, **characterized** in that the resolution enabling tag which additionally may act as a tracer tag is selected from a group consisting of labels recordable by fluorescence, luminescence, infrared absorption, electromagnetic properties, radioactivity and enzymatic activity.

11. The method according to claim 1, **characterized** in that the preset optional number of soluble oligonucleotide probes in the pool is more than one preferably more than five, most preferably more than ten.

12. The method according to claim 1, **characterized** in that the amount of the individual, quantitatively captured and released oligonucleotide probes is recorded with a fully or partly automized recording system, which is selected based on the applied resolution enabling tags.

13. The method according to claim 12, **characterized** in that the recording system is selected based on resolution enabling tags and comprises mass spectrometry, electrophoretic or chromatographic techniques.

14. The method according to any of claim 1, **characterized** in that the amount of the quantitatively recovered primer tagged probes are released and subsequently amplified and optionally tracer tagged before, during or after the PCR-reaction and thereafter recorded with a recording system selected based on the resolution enabling tags.

15. The method according to claim 1, **characterized** in that the oligonucleotide probes are DNA fragments, synthetic or recombinant oligonucleotide sequences or modified oligonucleotide sequences.

16. The method according to claim 1, **characterized** in that a comparative, quantitative assessment of variations in the amounts of individual polynucleotide sequences or organisms and subgroups thereof in a population or mixture of polynucleotide sequences by providing a set of multiple test kits, at least one test kit for each sample to be compared, each of said test kit comprising organized pools with identical soluble polynucleotide.

17. The method according to claim 16, **characterized** in that the individual test kits, wherein the resolution enabling tag is not a tracer tag, a set of multiple test kits is provided with tracer tags each being distinguishable from the other by the emitted signal.

18. The method according to claim 1, **characterized** in that the analyte sequences provided with an affinity tag are captured on the solid support before contacting it with the probe pools.

19. A test kit for determining the amounts or relative proportions of more than one individual polynucleotide sequence or subgroups thereof in a polynucleotide sequence mixture using a quantitative affinity aided solution hybridization, wherein the test kit comprises one or more organized pools with a preset optional number of soluble polynucleotide probes, which are distinguishable in order to provide resolution for recording of results, said pools being placed in an organized manner in their own vessels, which are separate or joined together, **characterized** in that said polynucleotide probes are oligonucleotide probes, which have approximately the same size and/or mass and are made distinguishable by providing said oligonucleotide probes with one or more

resolution enabling tags capable of changing the size or mass to electric charge ratio and thereby providing the oligonucleotide probes with different mobilities in the fractionation, separation or recording systems of the oligonucleotide probes without disturbing the hybridization and/or capturing reaction.

20. The test kit according to claim 19, **characterized** in that for the determination of dynamic variations in the amounts or relative proportions of polynucleotide transcripts or their subgroups in an individual organism, the soluble oligonucleotide probes are designed from species or group-specific oligonucleotide sequences hybridizing with selected more or less conserved or hypervariable region from intragenomic sequences specific for subgroups, species, subspecies of transcripts expressed in the organism.

21. The test kit according to claim 20, **characterized** in that the polynucleotide analytes isolated from the sample comprising the mixed target population comprise messenger RNA (mRNA).

22. The test kit according to claim 19, **characterized** in that for the determination of dynamic variations in the amounts or relative proportions of polynucleotide sequences representing individual organisms or subpopulations thereof in a target population, the soluble oligonucleotide probes are designed from species or group-specific oligonucleotide sequences hybridizing with selected more or less conserved or hypervariable region from intragenomic sequences specific for and/or representing different phylogenetic levels allowing the identification of subgroups, species, subspecies within the mixed target population.

23. The test kit according to claim 22, **characterized** in that the polynucleotide analytes isolated from the sample comprising the mixed target population comprise ribosomal RNA or DNA.

24. The test kit according to claim 19, **characterized** in that the resolution enabling tag may simultaneously act as a tracer, affinity or primer tag.

25. The test kit according to claim 24, **characterized** in that the resolution enabling tag is selected from a group consisting of oligonucleotide residues, amino acid residues, peptides, sugar residues, haptens and ligands.

26. The test kit according to claim 24, **characterized** in that the resolution enabling tag which additionally may act as an affinity tag and/or primer tag is an oligonucleotide

residue.

27. The test kit according to claim 24, **characterized** in that the resolution enabling tag which additionally may act as an affinity tag and/or a tracer tag is an amino acid or a peptide.

28. The test kit according to claim 24, **characterized** in that the resolution enabling tag which additionally may act as a tracer tag is selected from a group consisting of labels recordable by fluorescence, luminescence, infrared absorption, electromagnetic properties, radioactivity and enzymatic activity.

29. The test kit according to claim 24, **characterized** in that the preset optional number of soluble oligonucleotide probes in the pool is more than one preferably more than five, most preferably more than ten.

30. The test kit according to claim 24, **characterized** in that the soluble pools of oligonucleotide probes are placed in wells on a microtiter plate.

31. The test kit according to claim 19, **characterized** in that the oligonucleotide probes are DNA fragments, synthetic, recombinant or modified oligonucleotide sequences.

32. The test kit according to claim 19, **characterized** in that for a comparative, quantitative assessment of variations in the amounts of individual polynucleotide sequences or organisms and subgroups thereof in a population or mixture of polynucleotide sequences comprises a set of test kits, wherein at least one identical test kit having identical pools of oligonucleotide probes for each sample to be compared.

33. The test kit according to claim 32, **characterized** in that each individual test kits in the set of multiple test kits is provided with tracer tags, which are distinguishable from each other by the emitted signal.

34. Use of the test kit according to claim 19 for determining variations in the amount of more than one polynucleotide sequence in a mixture with the method according to claim 1 for assessing hygienic conditions and epidemiologic situations, effects of external stimuli or treatment modalities on a microbial population.

35. The use according to claim 34, wherein the external stimulus or treatment is selected from a group consisting of treatment with antibiotics or hygienic measures.

Abstract

The invention relates to a method and test kit for determining variations of more than one individual polynucleotide in polynucleotide mixtures and for assessing dynamic variations in a mixed population of organisms using affinity aided solution hybridization. The test kit comprises organized pools with same sized oligonucleotide probes made distinguishable by end-tailing with resolution enabling tags providing the probes with different sizes or masses to charge ratios. The resolution enabling tags may simultaneously act as tracer, affinity or primer tags. The probes are allowed to hybridize with affinity tagged analyte polynucleotides from the sample. The result is hybrids, which can be recovered on a separation aiding tool provided with a counterpart of the affinity tag. After the quantitative release of the probes, the individual probes can be recorded. The method and test kit are useful for determining hygienic and epidemiologic situations and evaluating the effect of antibiotic treatment and sanitary measures.

SEQUENCE LISTING

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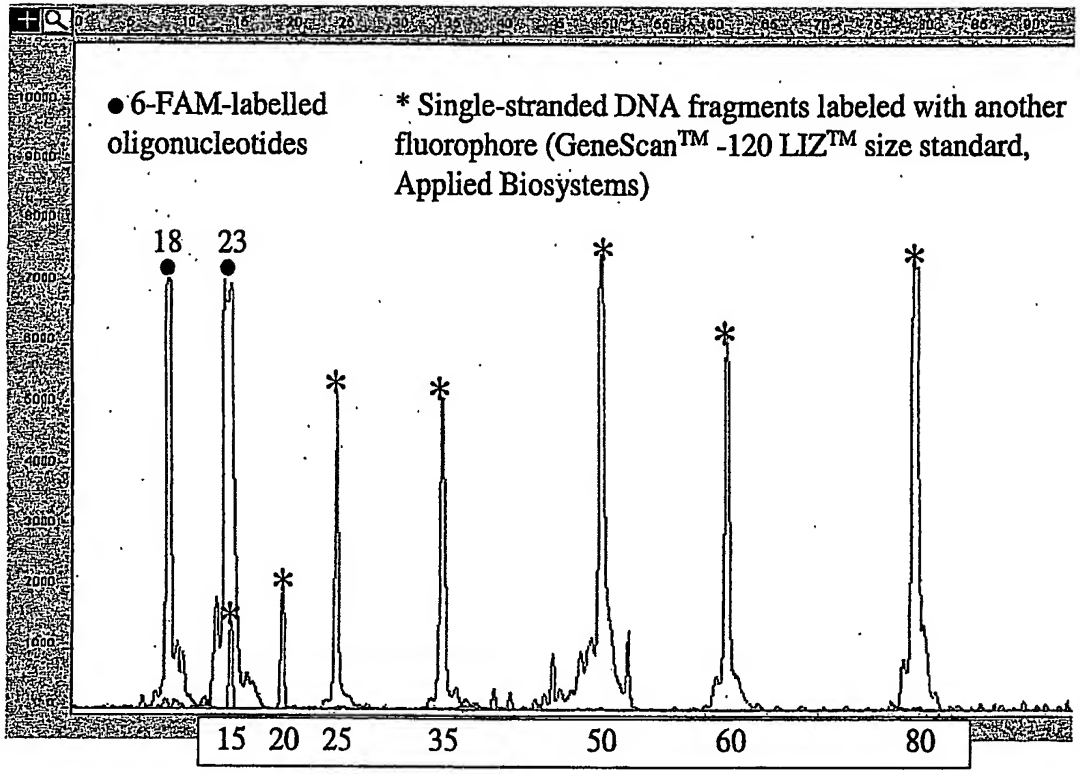
● 6-FAM-labelled oligonucleotides
* Single-stranded DNA fragments labeled with another fluorophore (GeneScan™ -120 LIZ™ size standard, Applied Biosystems)
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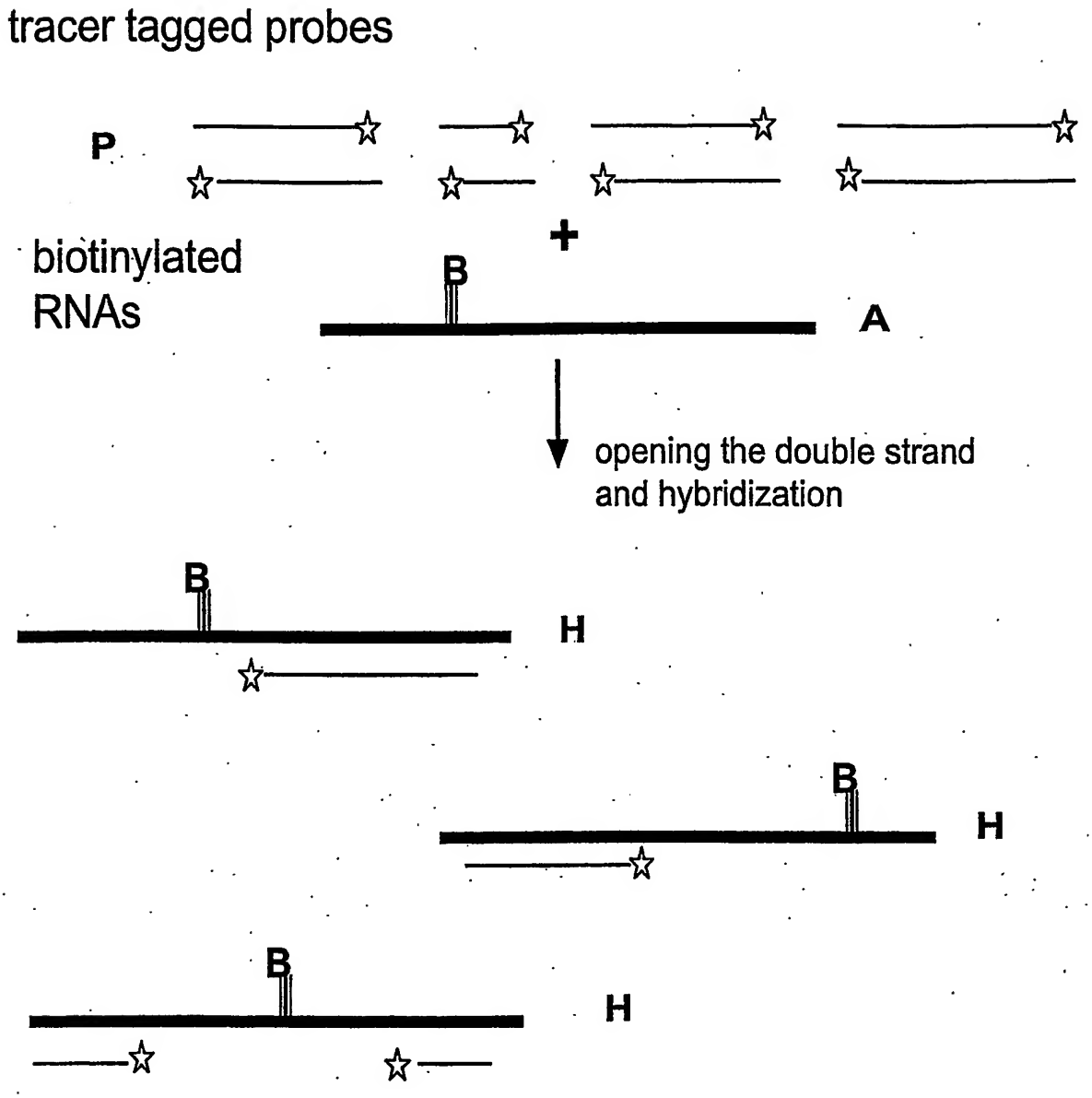
23
15
20
25
35
50
60
80

Fig. 1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B
A
opening the double strand
and hybridization
B
H
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B
H

Fig. 2A

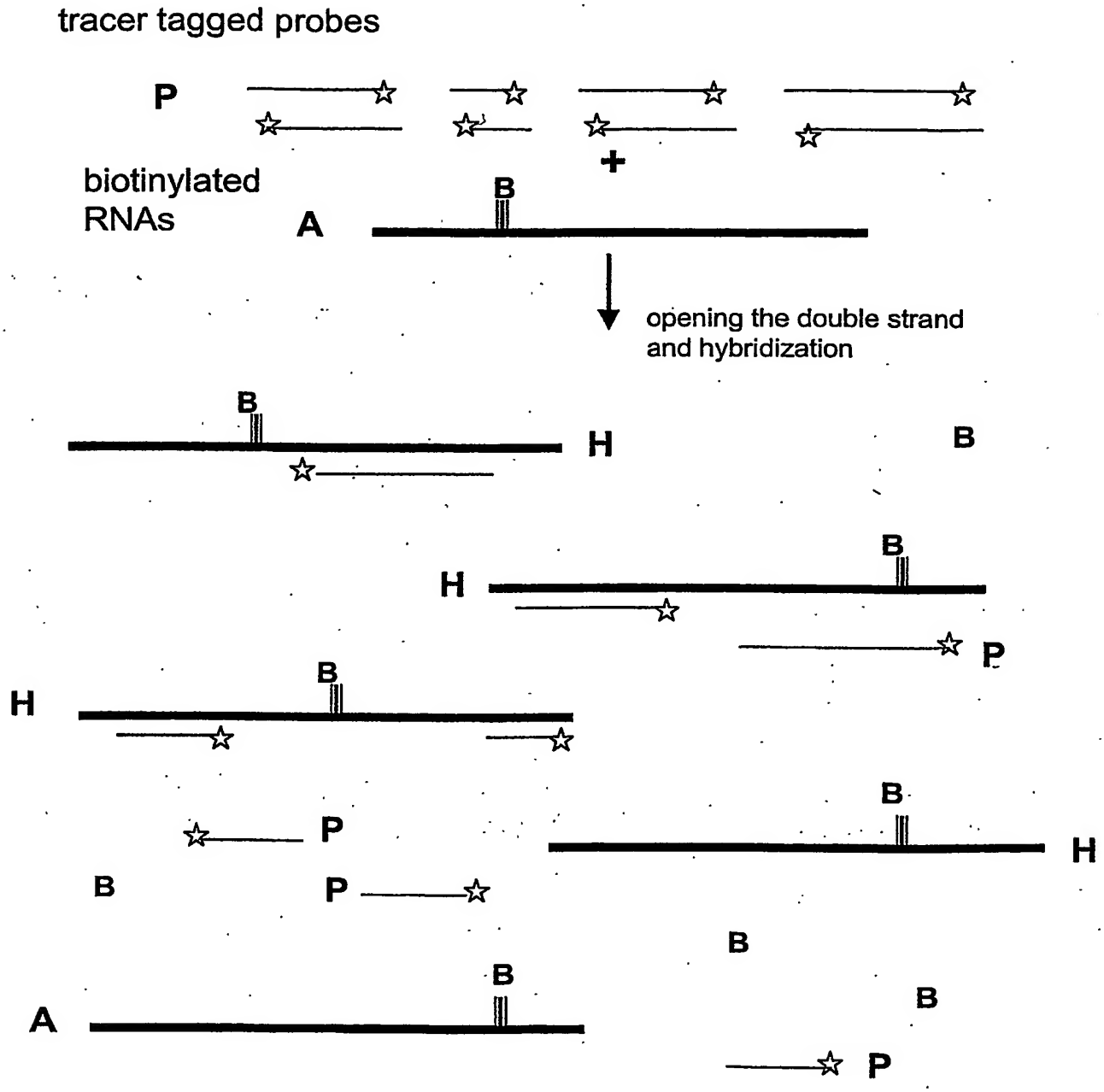
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Fig. 2B

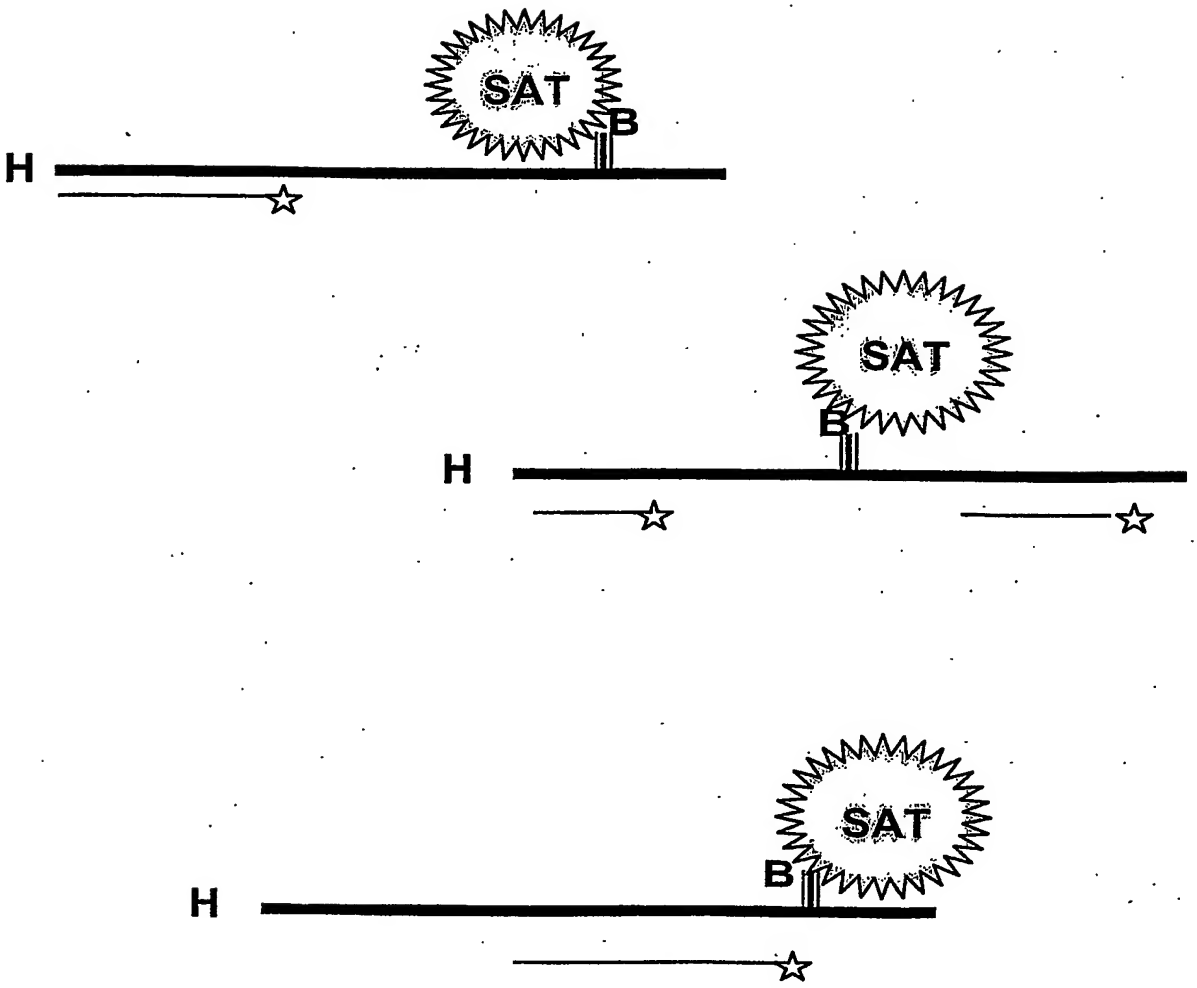


Fig. 3A

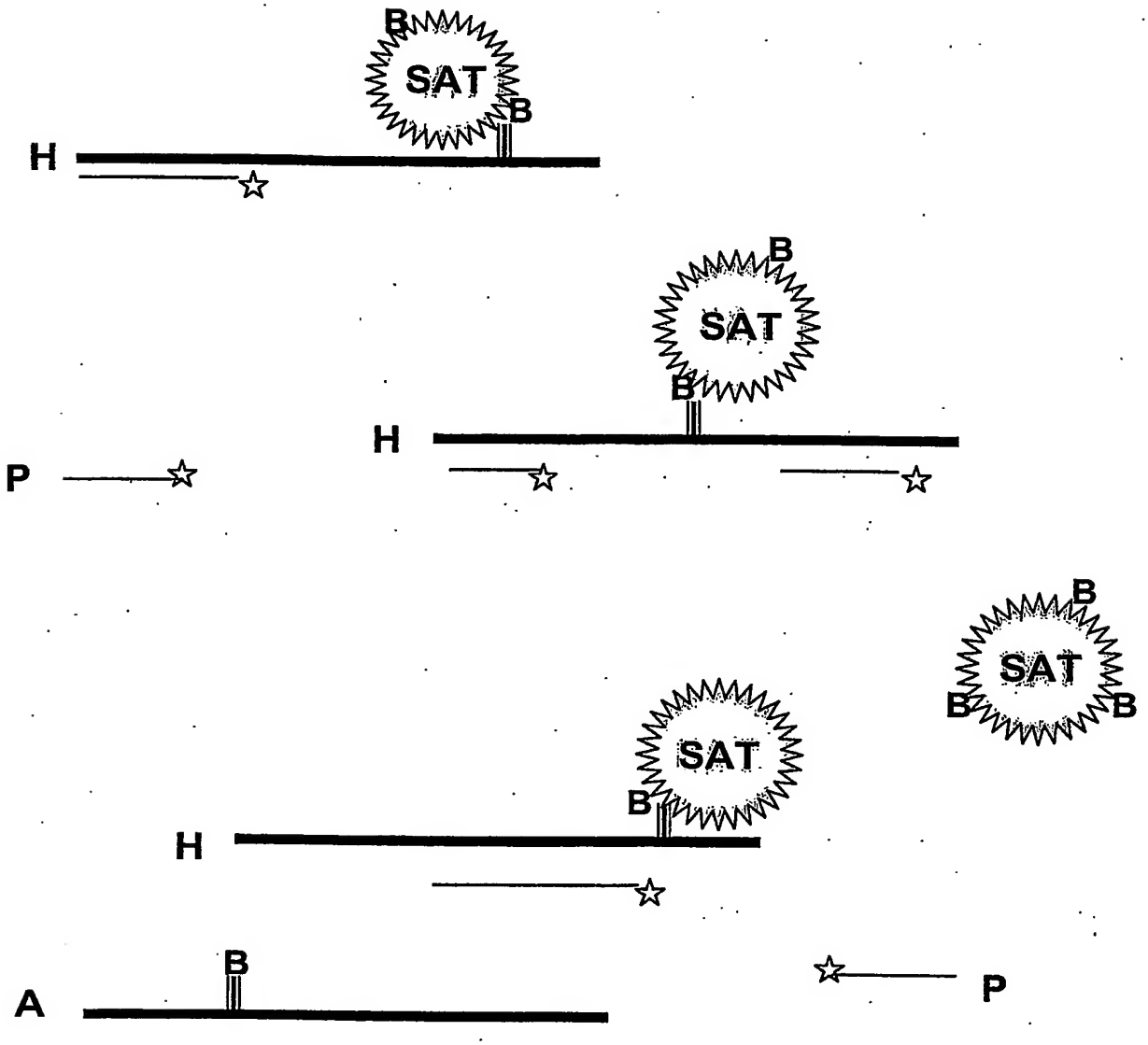
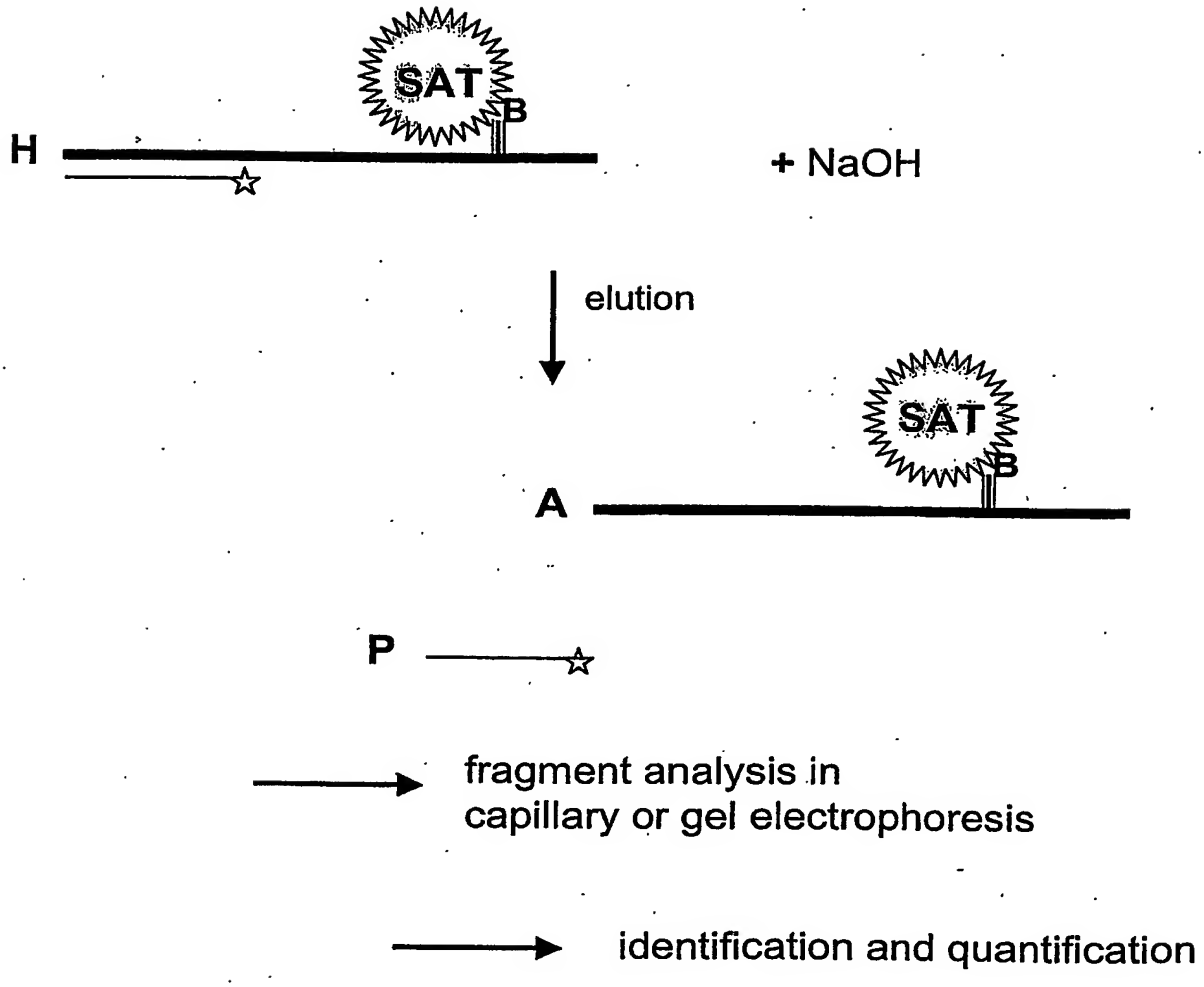


Fig. 3B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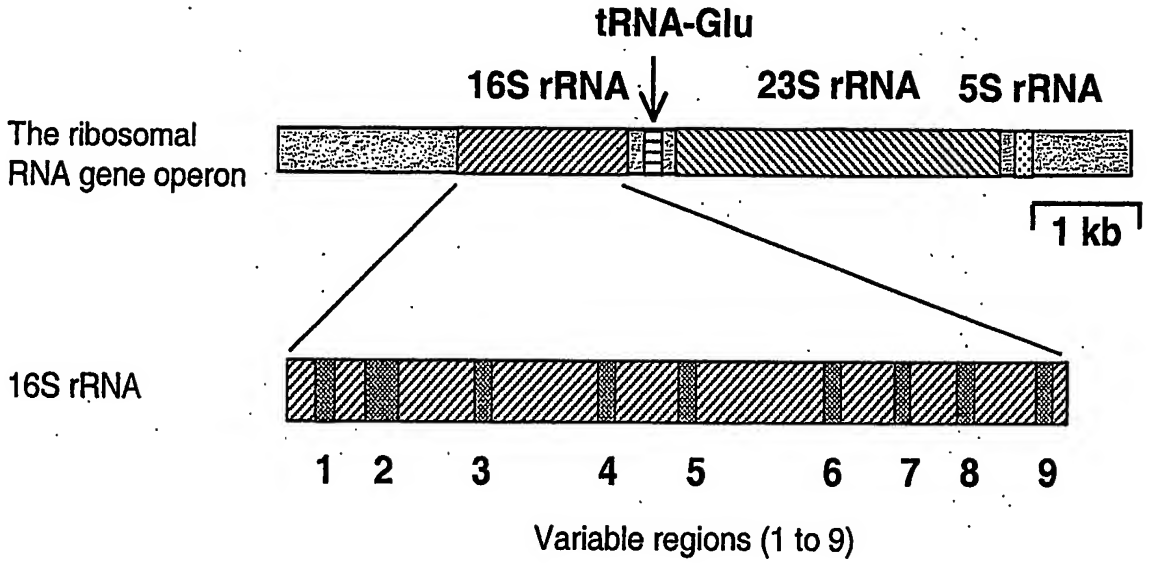
H
+ NaOH
elution
SAT
B
A
P
fragment analysis in
capillary or gel electrophoresis
identification and quantification

Fig. 4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1 2 3 4 5 6 7 8 9
Variable regions (1 to 9)

Fig. 5A

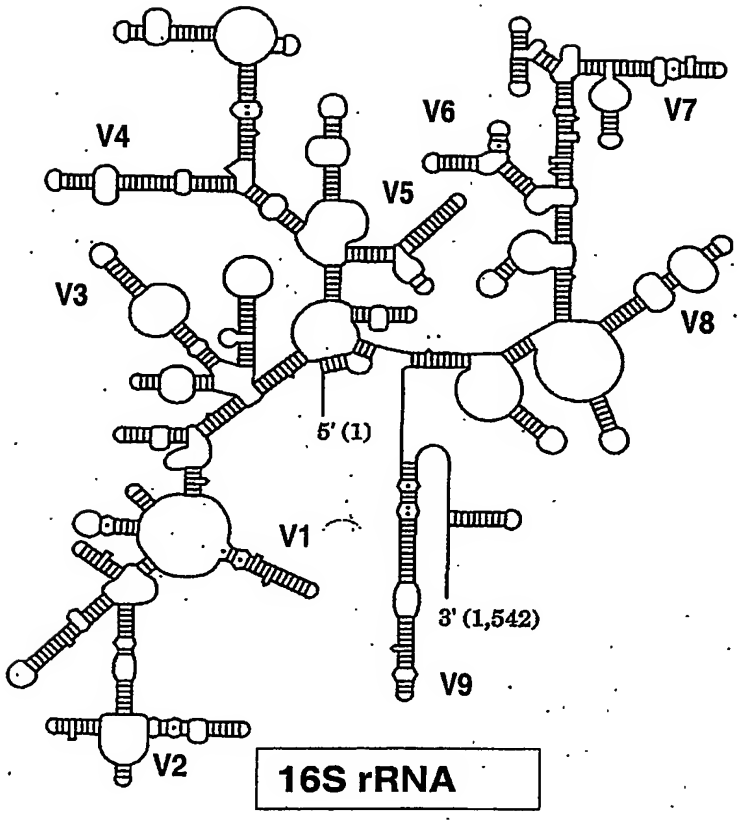
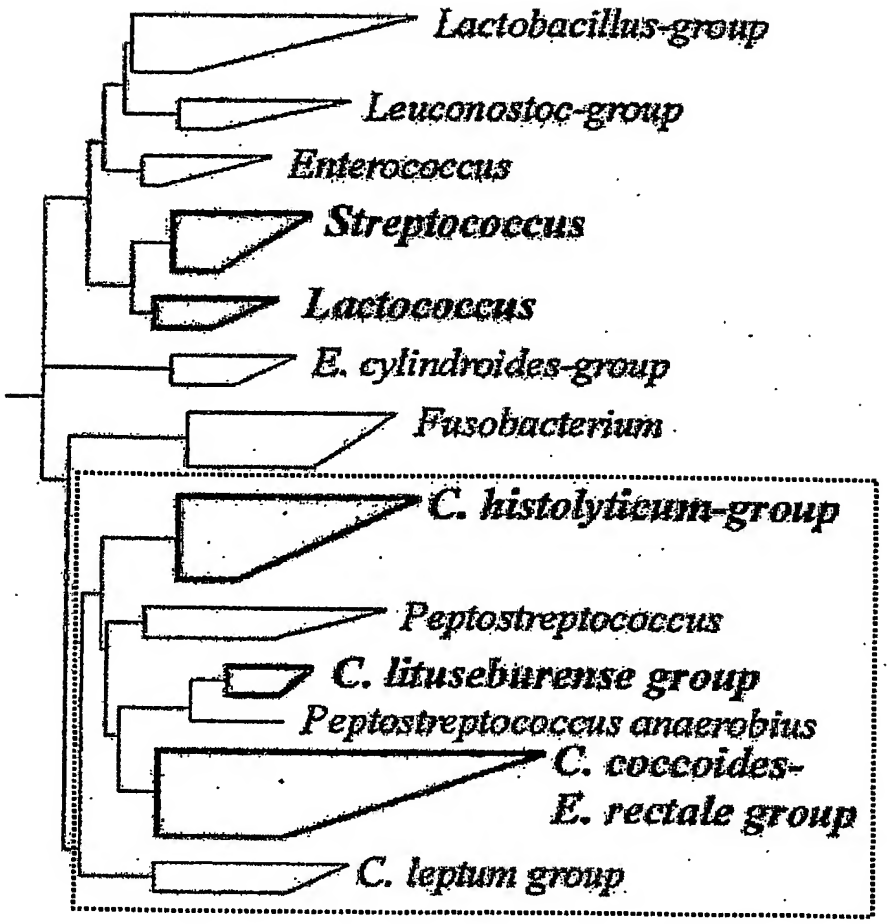


Fig. 5B



Adapted from Franks *et al*, 1998

Fig. 6

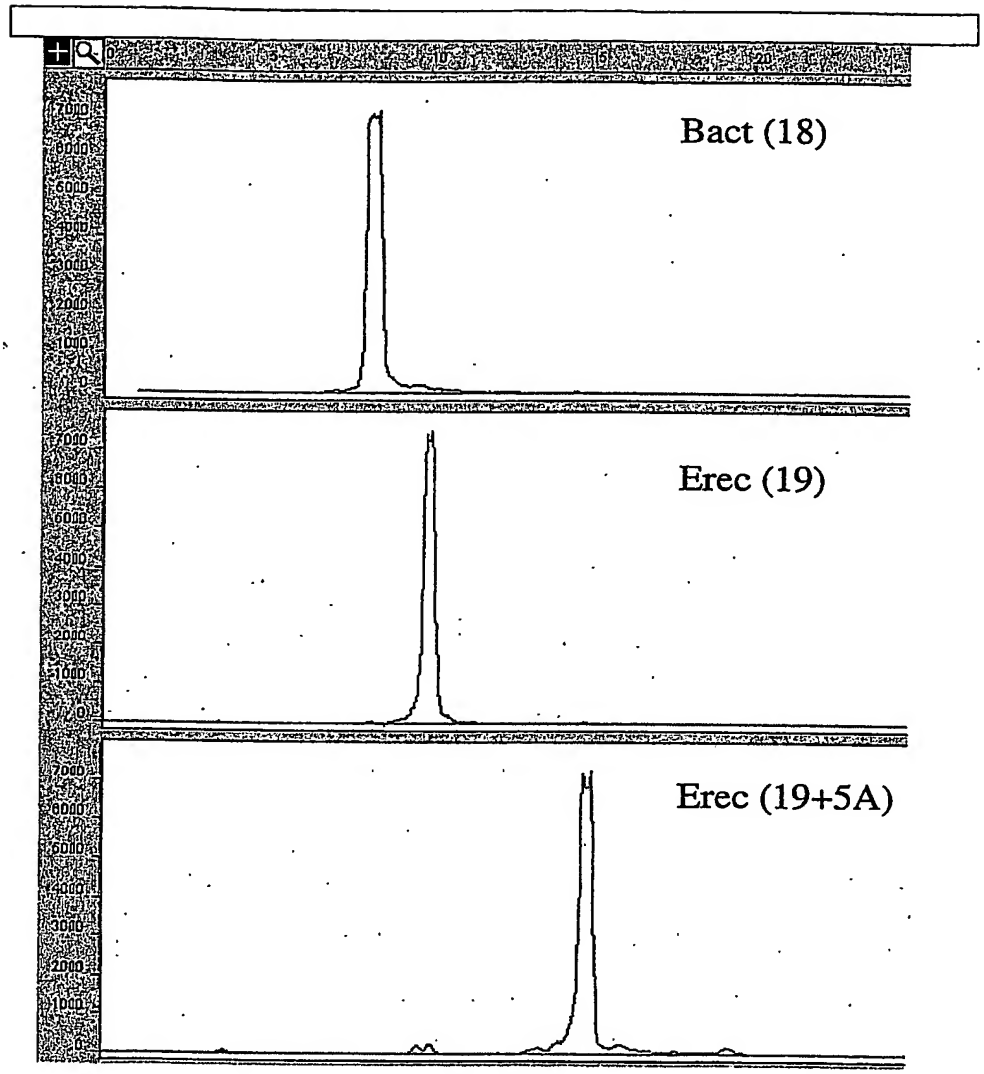
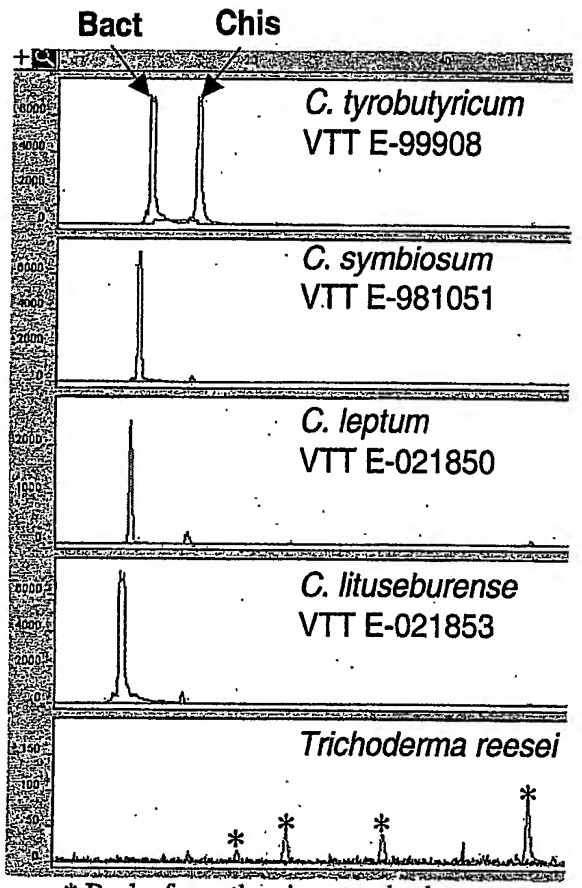


Fig. 7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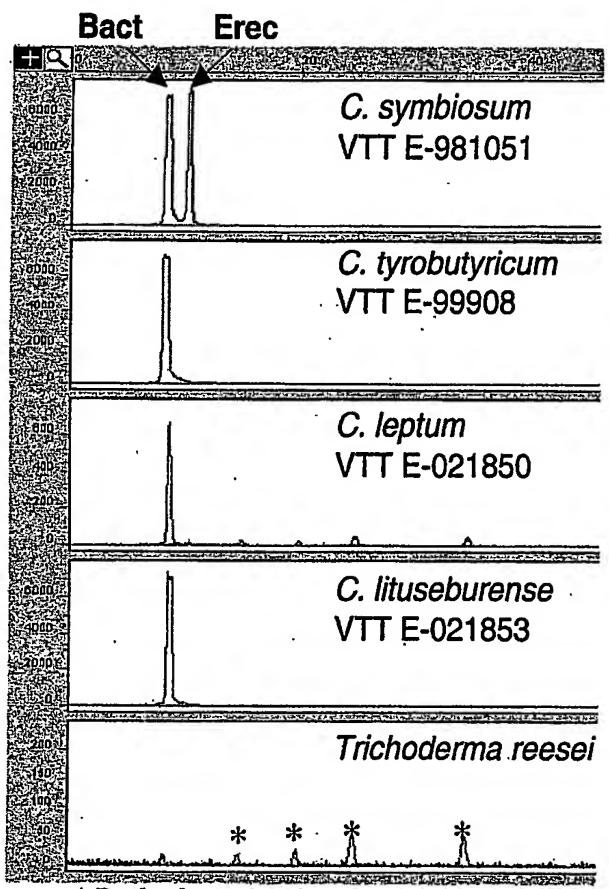
Trichoderma reesei
* Peaks from the size standard

Fig. 8A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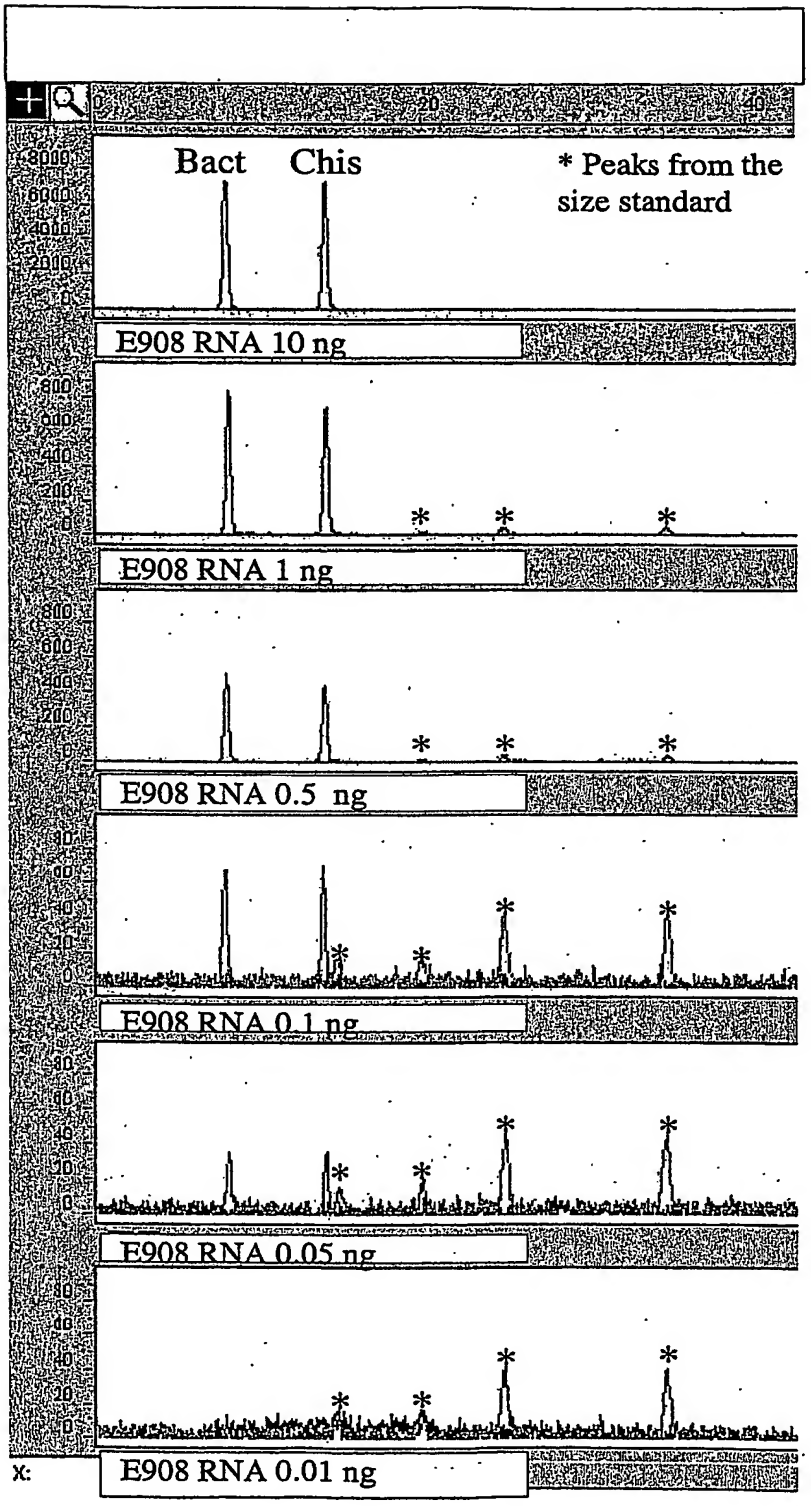
Trichoderma reesei
* Peaks from the size standard

Fig. 8B


Bact
Chis
* Peaks from the size standard
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E908 RNA 0.01 ng

Fig. 9

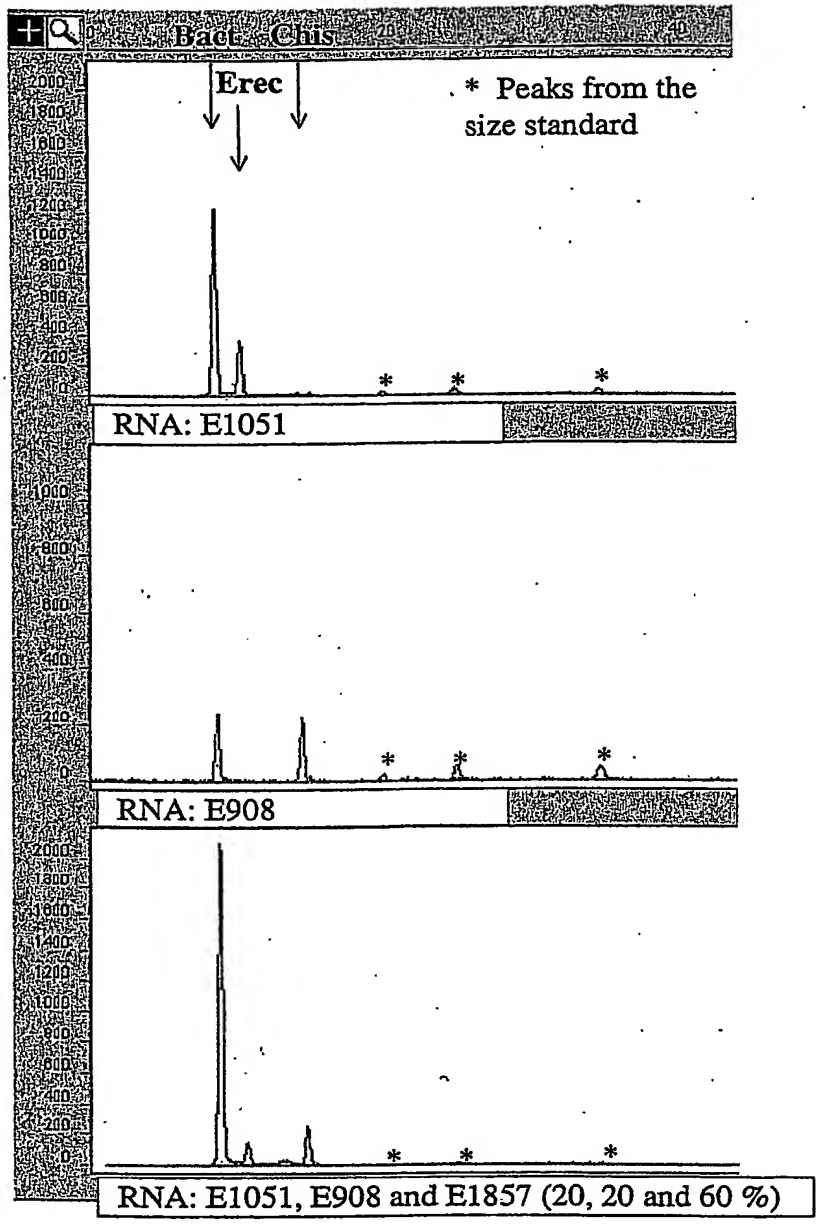
Bact Chis
Erec
* Peaks from the size standard
RNA: E1051
RNA: E908
RNA: E1051, E908 and E1857 (20, 20 and 60 %)

Fig. 10A

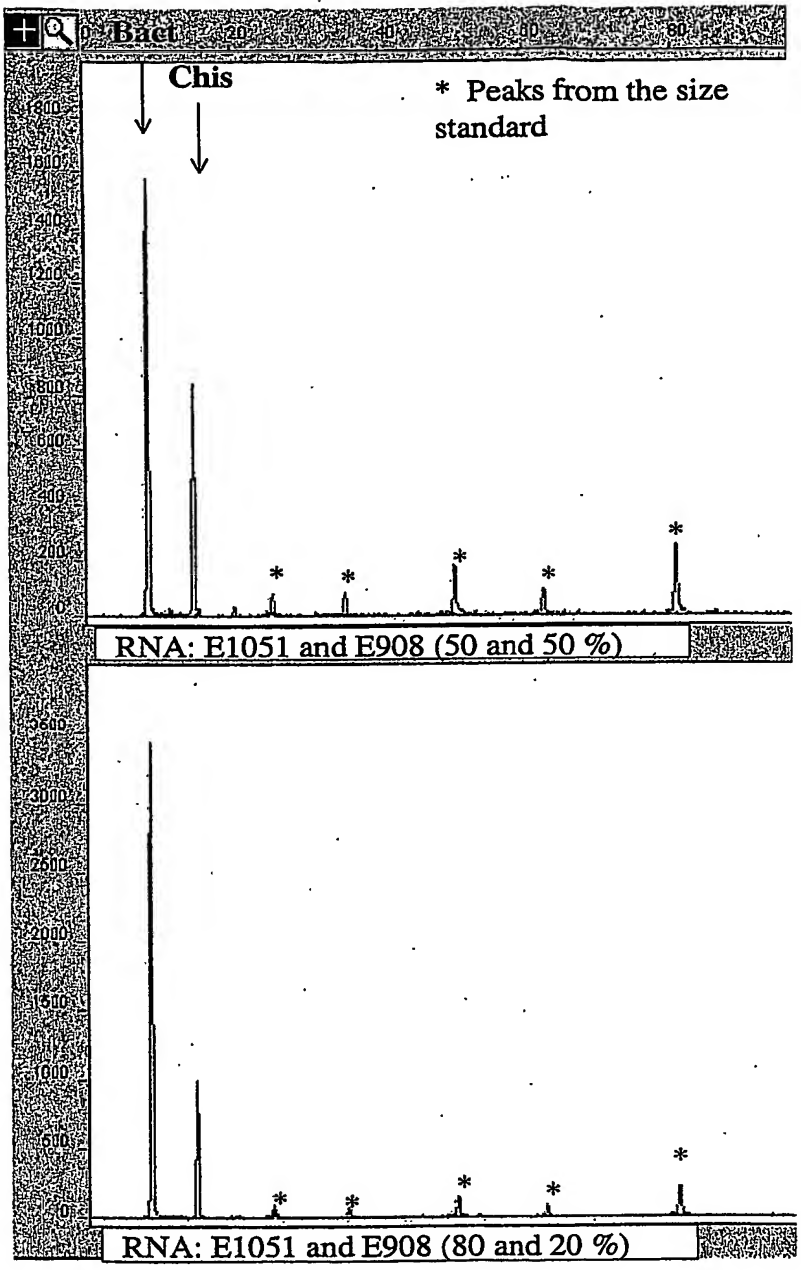
Bact
Chis
* Peaks from the size standard
RNA: E1051 and E908 (50 and 50 %)
RNA: E1051 and E908 (80 and 20 %)

Fig. 10B

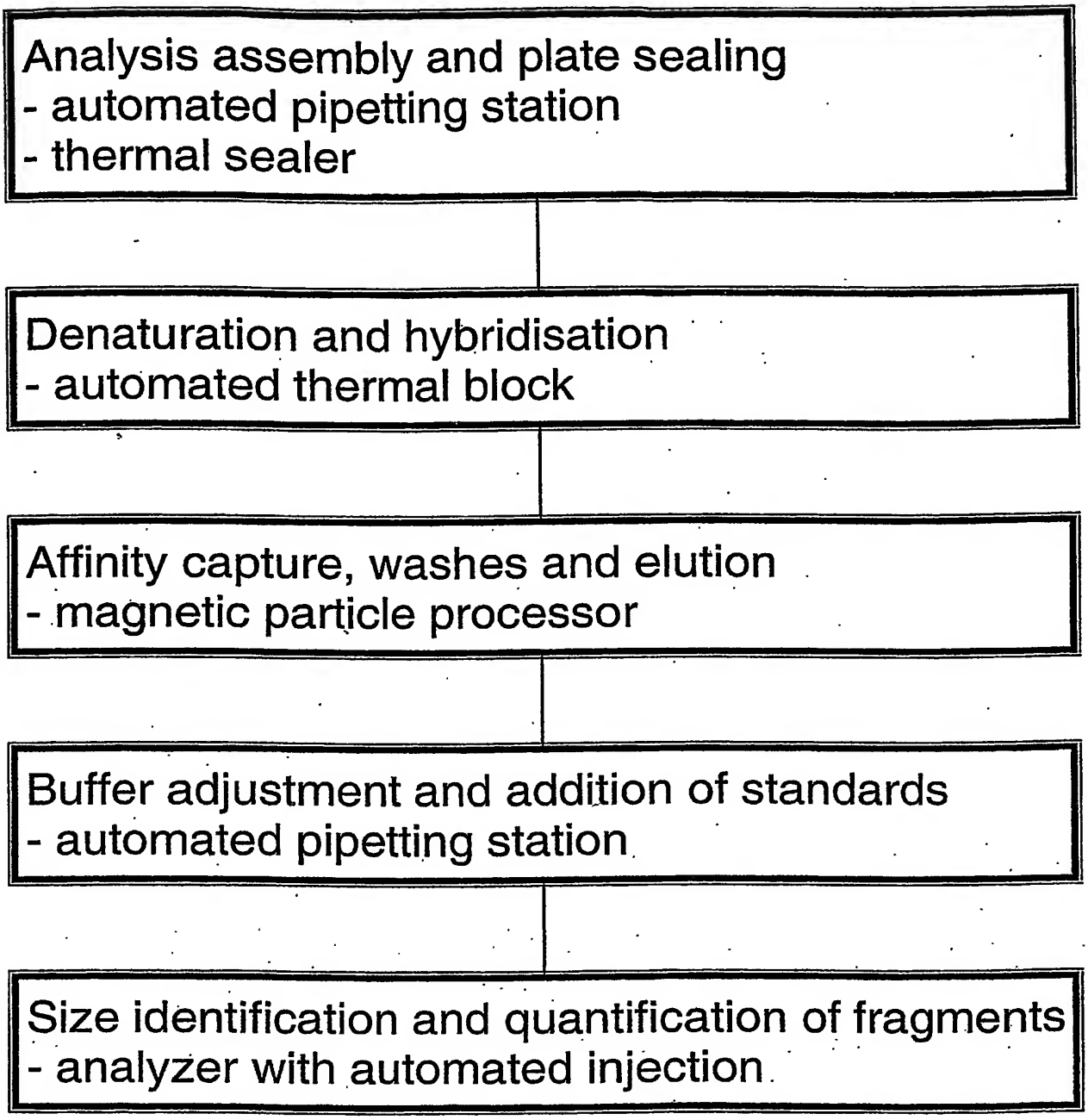
Analysis assembly and plate sealing
- automated pipetting station
- thermal sealer
Denaturation and hybridisation
- automated thermal block
Affinity capture, washes and elution
- magnetic particle processor
Buffer adjustment and addition of standards
- automated pipetting station
Size identification and quantification of fragments
- analyzer with automated injection

Fig. 11